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*Perspective*

# Intracellular Receptors and Signal Transducers and Activators of Transcription Superfamilies: Novel Targets for Small-Molecule Drug Discovery

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## Introduction

The proliferation and differentiation of mammalian cells is modulated by a number of specific signal molecules that regulate gene expression. Among these signals are (i) the steroid hormones (*e.g.*, glucocorticoids, mineralocorticoids, estrogens, progestins, and androgens), chemical messengers produced by the body in response to a variety of stimuli; (ii) small-molecule hormones including thyroid hormone, calcitriol (a vitamin $D_3$ metabolite), and the retinoids; and (iii) the cytokine superfamily of protein molecules that affect cells of the immune and other systems.

The mechanism of action of steroid hormones has been studied extensively over the last 25 years. The steroid hormones, which have similar fused ring skeletons, vary structurally with the placement of double bonds and the nature of their side chains. Dramatic differences in biological activity of these molecules are due to interactions with specific receptors capable of distinguishing minor differences in chemical structure. In contrast, nonsteroidal small-molecule hormones vary significantly in chemical structure; however, they utilize similar receptors to produce their biological effects. The receptors for the nonsteroidal small-molecule hormones include the retinoic acid (RA) receptor subtypes (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ), the vitamin D receptor, and the thyroid hormone receptor subtypes ($T_3$Rα and $T_3$Rβ and splice variants).

The definition of biochemical events that mediate signal transduction in response to steroid hormones and small-molecule hormones has advanced rapidly, beginning in 1985 with the first cloning of an intracellular receptor (IR).[1] Molecular biological techniques enabled subsequent cloning and characterization of receptors for each of the steroid and small-molecule hormones. This dramatically enhanced understanding of hormone action and led to a number of unifying insights concerning the receptors and their ligands. The IRs are closely related members of a protein superfamily[2] that have apparently diverged from a common ancestral gene.[3] The presence of a specific IR within a cell enables that cell to respond to the hormone cognate to that IR. The IRs share a common mechanism of action, since they in general remain latent inside target cells until exposed to their specific ligands, which activate them as transcription factors producing specific changes in gene expression.

Cytokines are a large and diverse family of circulating polypeptides produced by many different cell types. They include various types of interferons (*e.g.*, IFN-α, -β, -γ), the interleukins (*e.g.*, IL-6), the colony-stimulating factors (*e.g.*, granulocyte colony-stimulating factor, G-CSF), and growth factors (*e.g.*, epidermal growth factor, EGF). Individual cytokines act upon a variety of cell types. As polypeptides, cytokines cannot freely enter cells; they act by binding to specific cell surface receptors. The understanding of the biochemical events by which some of the cytokines achieve their distinctive biological effects has increased significantly since 1992. There is a surprising degree of underlying similarity in the pathways of cytokine signal transduction, explained by the discovery of a family of latent cytosolic proteins, termed signal transducers and activators of transcription (STATs),[4] that mediate signal transduction for the majority of the cytokines. This newly defined STAT protein superfamily acts to mediate specific changes in gene expression and consequently cell function following exposure to most cytokines.

Both the IRs and the STATs act as DNA-sequence-

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specific transcription regulators, selecting the genes expressed and modulating the level of their expression within a cell following exposure to a specific stimulus. The DNA sequence-specific factors that act to modulate gene transcription are collectively termed transcription factors and include the IRs and STATs, which exert their effects by binding to chromosomal DNA in a sequence-specific manner or by interacting with components of the transcription apparatus, the complex of RNA polymerases and accessory proteins that carry out the production of messenger RNA (mRNA). Transcription factors control the expression of specific sets of genes. Since the pattern of gene expression determines cell function, the control of gene expression is a central process in biology.

The concepts and terminology used to describe the transcription of eukaryotic genes will be briefly reviewed here, preceding a detailed discussion of the mechanics of the IR and STAT signal transduction pathways and their relevance to drug discovery. Transcription, the rate-limiting step in gene expression, is used by the cell as a primary point of regulation for subsequent events controlled by hormones and cytokines. The regulatory pathways involved in transcription are controlled by protein–protein and protein–DNA interactions.

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals is a complex process involving the concerted action of many proteins. RNA polymerase II is the enzyme responsible for the production of mRNA from genes in eukaryotes, as outlined in Figure 1.[5] It acts in combination with a number of transcription factors. These factors can be divided into three classes on the basis of their functions. First are the basal transcription factors, which are required for an unregulated, basal level of transcription by RNA polymerase II. Second are the DNA-sequence-specific transcription factors, which are required for regulated transcription of a subset of these genes. Lastly, the coactivators represent a newly discovered class of regulatory proteins that act in concert with sequence-specific and basal transcription factors to further modulate levels of transcription.

The regulatory region in the immediate vicinity of the transcription start site is termed the promoter and contains a number of core response elements. Response elements are specific nucleotide sequences that are recognized by and act as binding sites for transcription factors. The core response elements are usually located within several hundred base pairs of the transcription start site. The most common core response element among genes transcribed by RNA polymerase II is the 5′-TATAAA-3′ sequence (TATA box), recognized by a specific basal transcription factor. Other response elements commonly found in the promoter region and to which specific transcription factors bind include the 5′-GGGCG-3′ and 5′-CCAAT-3′ sequences. In addition, mammalian genes may contain particular combinations of positive or negative regulatory response elements that are uniquely arranged as to number, type, and spatial organization. These response elements are the binding sites for sequence-specific transcription factors that activate or repress gene expression for the gene downstream from that promoter. A response element that regulates the activity of the promoter from a distance and in an orientation-independent fashion is termed an enhancer or a silencer depending on whether it induces or represses gene expression. Both the IRs and STATs interact with response elements to control transcription.

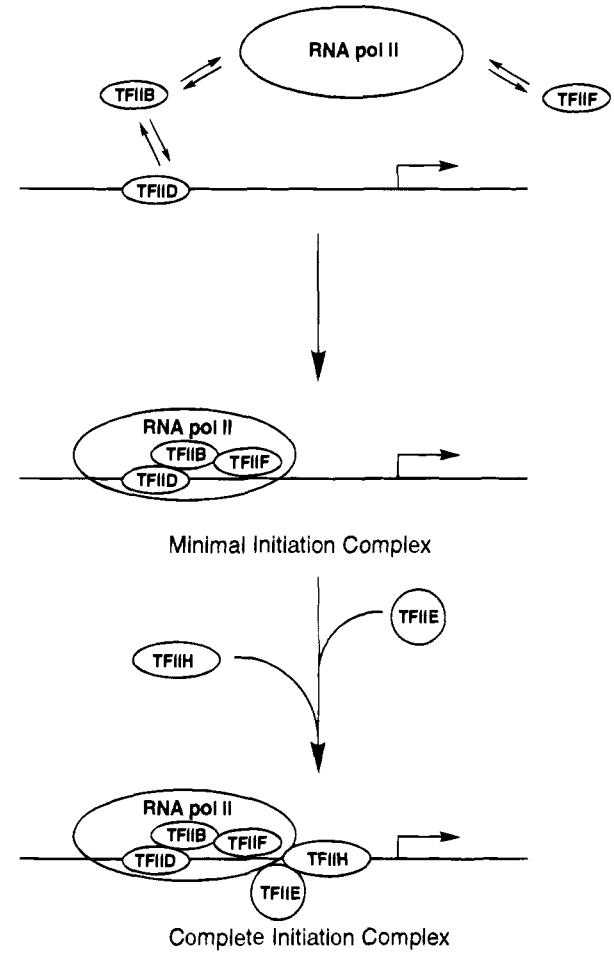


**Figure 1.** The cascade of events leading to transcriptional initiation. The first step in the initiation process requires binding of TFIID to the TATA box of the promoter. After TFIIB binds to TFIID on the DNA, RNA polymerase II and TFIIF are recruited to the complex of proteins at the transcription initiation start site. Transcriptional initiation also requires the presence of TFIIE and TFIIH to yield a complete initiation complex.

Initiation of transcription requires that the basal transcription factors, by interaction with core response elements, form an initiation complex, the active assemblage of RNA polymerase II and accessory proteins required to start RNA synthesis. A schematic representation of initiation complex formation is shown in Figure 1. The first step in the assembly of the initiation complex is the binding of the transcription factor D for RNA polymerase II (TFIID)[6] to the TATA box. TFIID is a multiprotein complex that consists of the TATA binding protein (TBP) and TBP associated factors. TFIID acts as a binding site for TFIIB. Once bound, TFIIB is able to recruit RNA polymerase II and TFIIF to the transcription start site. The complex formed by the association of these factors is stable; however, subsequent association of transcription factors TFIIE and TFIIH is required to complete formation of the transcription initiation complex to begin mRNA production.[5,7]

Sequence-specific transcription factors modulate the formation of the initiation complex and thus control the frequency of transcription of a specific subset of genes

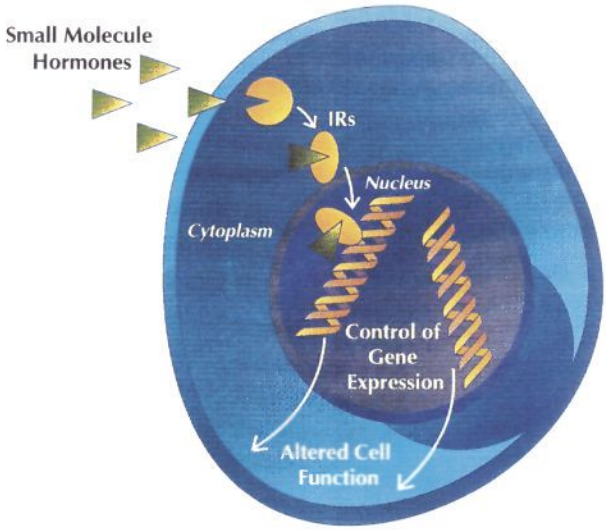


**Figure 2.** Control of gene expression through the intracellular receptor signaling pathway. In the IR pathway, small-molecule ligands diffuse into the cell and bind to the appropriate receptor. This leads to a conformational change of the receptor that causes dissociation with proteins such as hsp90 that are associated with the inactive form of the receptor and allows receptor binding to a specific response element in the promoter of a gene controlled by that hormone. Binding of an activated IR protein to its response element modulates the transcription of the downstream gene and, thus, translation of the gene product.

by RNA polymerase II. These transcription factors may act (i) by binding to sequence-specific response elements (enhancers or silencers) within the DNA or (ii) *via* direct protein−protein interaction with basal transcription factors within the initiation complex.

Recently, coactivators have been identified as a third class of transcription factors. It has been proposed that these function as physical links between the sequence-specific transcription factors and one or more components of the initiation complex. The TFIID complex, for example, contains several proteins that are tightly associated with the TBP and display some coactivator function. These proteins may be unnecessary for basal level transcription but essential for stimulation of transcription by sequence−specific transcription factors.

The remainder of this review will focus on two families of sequence-specific transcription factors: (i) the IRs, the transcription factors through which steroid hormones and small-molecule hormones control gene expression, and (ii) the STATs, the transcription factors through which many cytokines and growth factors control gene expression.

Information regarding hormonal and cytokine signal transduction that has delineated the underlying principles, properties, and biological roles of IRs and STATs will be discussed. Important implications for new drug discovery approaches, targets, and tools with the potential to yield breakthrough small-molecule drugs mimicking or blocking hormone and cytokine actions will be highlighted.

## Intracellular Receptor Function

The sequence of events involved in IR signal transduction is shown in Figure 2. Briefly, the non-peptide hormones, such as estrogen or RA, are sufficiently lipophilic to diffuse freely through the cell membrane without the need for specialized transport systems. A number of these hormones interact with plasma and intracellular binding proteins that show varying degrees of specificity; however, the actual mediators of hormonal signal transduction are the IRs. The intracellular receptors, proteins with molecular masses that range from approximately 55 to 90 kDa, have a characteristic domain structure. They are located within the cell, but are not cell membrane associated. Once a hormone enters the cell, it binds to its IR with high affinity, resulting in a conformation change in the receptor, activating the IR as a transcription factor.

In the absence of bound ligand, inactive steroid hormone receptors are sequestered in cells in a complex with the heat shock proteins hsp-90, hsp-70, and p59.[8] Additional proteins, such as YDJ1, also appear to influence the activation of steroid hormone receptors.[9] The cellular localization of the unliganded complex (cytoplasmic or nuclear) remains controversial.[10] The conformational change that occurs in the receptor as a consequence of hormone binding results in the dissociation of the IR from the heat shock proteins and release of the monomeric receptor molecule and its ligand from the complex. In contrast to the steroid hormone receptors, the inactive small-molecule hormone receptors do not appear to interact with heat shock proteins and, in the absence of hormone, are located in the nucleus.[11] The binding of hormone also results in conformational changes in these receptors and in their subsequent activation. The exact nature of these conformational changes is not known. However, for some of the IRs it can be shown to involve alteration in the accessibility of the IR's C-terminal region, detectable either with immunological reagents[12] or by determination of ligand modulation of proteolytic susceptibility *in vitro*.[13]

There are approximately 100000 genes expressed in all cells, with 10000−20000 expressed in a single cell. Of these, only a few hundred genes in any cell are regulated by IRs. Ligand-activated IRs exert their effects by binding directly to specific chromosomal enhancer sequences termed hormone response elements (HREs) that are located within the regulatory regions of target genes.[14,15] Once bound to HREs, the activated receptor increases the transcriptional activity of the adjacent promoter, resulting in optimal expression of the target gene. Each HRE is made up of two approximately hexanucleotide half-sites separated by a variable number of nucleotides. The sequence of the half-sites and the number, but not the sequence, of the spacing nucleotides are key determinants of the specificity of IR interaction. HREs differ in their nucleotide sequences as well as the orientation and spacing of their half sites. Comparison of the sequences of the HREs from different hormone responsive genes indicates that a similar motif is used by each of the receptor subfamilies (Table 1).

Recent evidence shows that the IRs can associate to form homodimers, heterodimers, and possibly other oligomeric receptor species.[16,17] These dimers may bind to inverted repeats, direct repeats, or everted repeats. It is generally believed, in the case of the glucocorticoid receptor (GR),[1] estrogen receptor (ER),[18] progesterone receptor (PR),[19,20] androgen receptor (AR),[21−23] and mineralocorticoid receptor (MR)[24] that the active receptor species are homodimers. For many of the other IRs, including thyroid hormone receptor (TR),[25] RA receptors

**Table 1.** Hormone Response Elements

| Receptor | | Example of HRE | Consensus Sequence |
|---|---|---|---|
| GR, MR, | MMTV | GTTACA AAC TGTTCT | GGTACANNN TGTTCT |
| AR & PR | TO | TGCACA GCG AGTTCT | " |
| | TAT | TGTACA GGA TGTTCT | " |
| ER | cVit | GGTCA GCG TGACC | GGTCANNN TGA/TCC |
| | rPrl | TGTCA CTA TGTCC | " |
| RXR | rCRBPII | AGGTCA C AGGTCA | AGGTCA N AGGTCA |
| VDR | hOST | GGGTGA ACG GGGGCA | AGGTCANNN AGGTCA |
| TR | hMHC | AGGTGA CAGG AGGACA | AGGTCANNNN AGGTCA |
| RAR | hRARβ | GGTTCA CCGAA AGTTCA | AGGTCANNNNN AGGTCA |

MMTV, mouse mammary tumor virus; TO, tyrosine oxidase; TAT, tyrosine aminotransferase; cVit, chicken vitellogenin; rPrl, rat prolactin; rCRBPII, rat cellular retinol-binding protein type II; hOST, human osteocalcin; hMHC, human cardiac myosin heavy chain; hRARβ, human RARβ. ←→ : inverted repeat of half-site. → →: direct repeat of half-site.

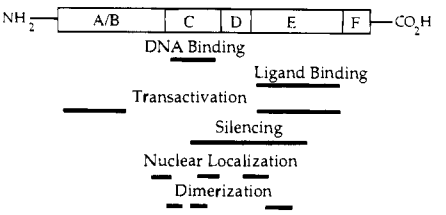


**Figure 3.** The domain structure of the intracellular receptors. The DNA-binding domain (DBD) corresponds to the C region and the ligand-binding domain (LBD) corresponds to the E region. These critical domains are restricted to defined segments of the protein. However, the segments of the receptor responsible for transactivation, nuclear translocation, and dimerization are not restricted to single defined domains, but are found in multiple regions of the receptor. The region marked D is a short region known as the hinge region that connects the DBD and the LBD and has functional activity as shown in the figure. The DBD contains nine cysteine residues enabling the coordination of two zinc ions and the formation of two zinc fingers. The first zinc finger contains the P box responsible for the sequence specificity of the IR; the second zinc finger of the DBD contains the D box, which discriminates between similar response element sequences with different spacing between the half-sites of the response element.

(RARs),[26,27] vitamin D receptor (VDR),[28] and a number of the orphan receptors, the functional transcription factor is a heterodimer formed with a member of the retinoid X receptor (RXR)[29,30] subfamily.

The idea that the receptors can only bind to HREs as dimers is being reexamined since the estrogen receptor appears able to bind as a monomer to a single half-site of either the estrogen response element or the thyroid hormone response element.[31,32] Further, two recently described proteins with significant primary sequence homology to members of the IR superfamily, nerve growth factor I-B (NGFI-B)[33,34] and steroidogenic factor-1 (SF-1),[35] also bind to half-sites as monomers. In each case, the HRE is an extended estrogen response element with extra 5′ nucleotides. Although not clearly understood, the selection of the DNA-binding mode appears to be determined by response element type, promoter context, and relative levels of the pertinent IRs.

## Intracellular Receptor Structure

The cloning and sequencing of cDNAs for the IRs and comparison of their deduced amino acid sequences show that the superfamily members are modular in structure.[2,36] Sequence data and functional analysis show the IRs to consist of six discrete subdomains, A–F (Figure 3). Three of these domains have been described in detail: (i) the DNA-binding domain, which is highly conserved and provides specific binding to the HRE, (ii) the ligand-binding domain located C-terminal to the DNA-binding domain, which provides a hydrophobic pocket for the binding of the ligand but also contains a number of other functionally important regions, and (iii) the C- and N-terminal transactivation domains, which are more variable in sequence.

**I. DNA-Binding Domain.** The DNA-binding domain, which is usually centrally located in the primary sequence of an IR, is composed of 68 amino acid residues. Of those 68 amino acids, 20 are invariant and determine the generalized DNA-binding structure.[37] Confirmation that this region is the DNA-binding domain was obtained using XFACS,[38] 2D NMR,[39,40] and X-ray crystallography.[41] The DNA-binding domain is highly basic and contains nine cysteine amino acid residues. The presence of the cysteine residues enables the coordination of two zinc ions and the formation of two "zinc fingers". Selection of the specific HRE to which the receptor binds is determined by the three amino acids at the base of the first finger.[15] Mutational analysis of the estrogen receptor showed that conversion of these three amino acid residues to the corresponding residues from the glucocorticoid receptor results in a mutant estrogen receptor that binds the glucocorticoid response element.[42] The finger segment encompassing these residues, responsible for sequence specificity or selectivity of DNA binding, is termed the Proximal or P box.[43,44] The sequence spanning cysteine residues 5 and 6, in the second zinc finger, is responsible for discriminating between response elements with similar sequences but different half-site spacing. This sequence of five amino acid residues is called the Distal or D box.[45] For example, the D box in the RXR recognizes a one nucleotide spacing between the response element half-sites (5′-AGGTCA n AGGTCA-3′), while the RAR D box recognizes half-sites with five spacing nucleotides (5′-AGGTCA nnnnn AGGTCA-3′).

**II. Ligand Binding Domain.** The C-terminal or E region of the IRs is approximately 25 kDa and contains the ligand-binding domain (LBD), which determines the ligand-binding specificity of each receptor.[2,36] Proteolytic mapping of the receptor indicated that a portion of the D region is necessary for binding of ligand with maximal affinity.[12] The hormonal ligands generally bind to their cognate IRs with affinity constants ($K_d$ values) on the order of 1 nM. Upon binding hormone, the receptor is thought to undergo a major conformational change that results in its activation.[12] The crystal structure of the RXRα ligand binding domain has been reported.[46] However, since the crystal structure was determined in the absence of a ligand, the conformational changes induced by hormone binding have not yet been clearly defined. The ligand-binding domain additionally contains regions allowing (i) the dimerization of the receptor monomers,[45,47,48] (ii) the interaction of selected IRs with heat shock proteins,[8] (iii) nuclear translocation signals,[49] and (iv) one of

several transcriptional transactivation domains of the receptor. The dimerization[50] and nuclear translocation[51] signals that have been mapped to the ligand binding domain are dependent upon the binding of hormone for their action. Nuclear translocation and dimerization signals are also present in the DNA-binding domain, but their action is hormone independent. Analysis of the region of the ligand binding domain that contains the dimerization signal has revealed a heptad repeat of hydrophobic residues that are highly conserved within the IR superfamily. This observation suggests that the nuclear hormone receptors dimerize via a leucine-zipper type mechanism.[52]

**III. Transactivation Domains.** Transactivation domains are located in both the N-terminal and C-terminal regions of the receptors. Transactivation achieved with the DNA-binding domain alone represents a small portion of the total activity of the receptor. Deletion of the E regions of GR and ER, however, abolishes both hormone-binding and transactivation functions of the receptors. Since nuclear localization is a prerequisite for transactivation, it is difficult to assess the influence of the E region independent of its nuclear translocation function. In addition, at least in the case of the estrogen receptor, transactivation has been shown to be dependent upon the binding of hormone.[53] The situation is further complicated by the possibility that dimerization of the receptor may be necessary for efficient transactivation. However, careful mutational analysis, together with experiments involving chimeric genes, has enabled identification of specific regions that contain the transactivation functions for a number of the IRs. The transactivation domains, located N-terminal and C-terminal to the DNA-binding domain, have been termed transactivation unit 1 and 2, respectively (Tau 1 and Tau 2), in GR,[54] transactivation function 1 and 2 (TAF-1 and TAF-2) in ER,[55] and activation function 1 and 2 (AF-1 and AF-2) in PR.[56] The Tau 1, TAF-1, and AF-1 activation domains are located within the region marked A/B in Figure 3; the second activation domain of these receptors can generally be found in the LBD. The retinoid receptors, RARs and RXRs, have also been reported to have two domains responsible for transactivation, one each in the N-terminal and C-terminal regions.[57]

## Application of Intracellular Receptor Technology

Elucidation of the mechanism of IR-mediated transcriptional activation has enabled the development of high-throughput assays to detect novel small molecules that act as agonists, antagonists, or partial agonists of the IRs.[58] These assays can reveal the consequences of the interaction of any compound with any IR, using the cloned human IR cDNAs. These assays, termed cotransfection assays, are capable of detecting the functional effects on gene expression of small molecules that interact with specific IRs in mammalian cells. As shown in Figure 4, a gene for an IR that is a potential drug target is introduced by transfection (a procedure to induce cells to take up foreign DNA) into a mammalian cell lacking endogenous IRs of the type being studied. The introduced receptor cDNA directs the recipient cells to synthesize the receptor protein. A second gene under the transcriptional control of the appropriate HRE is also introduced by transfection (*i.e.*, cotransfected) into the same cells together with the IR gene. The protein product of this second gene functions as a reporter for the transcription-modulating activity of the receptor protein at its HRE. Thus, the reporter acts as a surrogate for the products normally expressed by genes under the control of the target receptor and its natural hormone. The reporter gene is chosen to encode a protein product that can be readily detected and quantified. Enzymes are useful reporters because they can often be assayed easily and, under the appropriate assay conditions, the rate of the reaction they catalyze directly reflects the amount of enzyme present. Firefly luciferase is an example of a frequently used reporter. The promoter that controls expression of the luciferase cDNA in the reporter plasmid is constructed to contain the appropriate HRE so that expression is under hormonal control at the level of transcription.

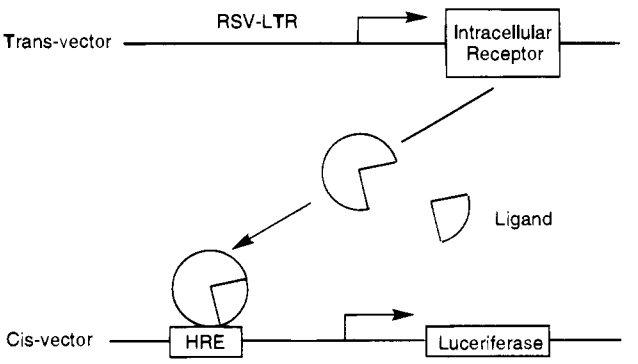


**Figure 4.** The cotransfection assay. Two plasmids are cotransfected into the appropriate cell background. One plasmid constitutively expresses an intracellular receptor. In the presence of ligand, the receptor acts in *trans* to bind to its HRE (the *cis* element) and activate transcription of the downstream reporter gene. The reporter is generally an easily detected enzyme such as chloramphenicol acetyl transferase (CAT) or luciferase.

The cotransfection assay can be used to detect agonists for the target IR. A day after exposure to an appropriate agonist ligand in the medium bathing the transfected cells, an increase in reporter activity can be measured in cell extracts, reflecting ligand-dependent, IR-mediated increases in reporter gene transcription.

The cotransfection assay can also be used to detected the activity of small molecules that antagonize the activity of an agonist ligand for any IR. To detect antagonists the assay is carried out in the presence of a constant concentration of a known agonist sufficient to induce a constant reporter signal. Cells incubated in the presence of increasing concentrations of an antagonist will display progressive decreases in reporter signal. The cotransfection assay is therefore useful to detect both agonists and antagonists of specific IRs.

## Current Therapeutics

Hormone-based therapies have been part of clinical medicine for over a century. Initially, extracts of endocrine glands were used as replacement therapies to supplement patients with glandular deficiencies.

Subsequently, the hormones themselves, purified from such extracts, were administered for similar uses. As the structures of the hormones were determined, chemically-synthesized versions replaced many of the naturally-derived hormone drugs. Subsequently, chemical analogues of these were synthesized and tested in animals to find compounds with improved therapeutic profiles relative to those of the hormones. Hormone agonists in clinical use include estrogens, anti-inflammatory glucocorticoids such as cortisone and dexamethasone, thyroxine, vitamin $D_3$ (a precursor to calcitriol), various progestins, and estrogens used in oral contraception, and vitamin A metabolites.

The sex steroids drive the growth and function of the tissues of the reproductive tract and breasts. Malignancies arising in these organs, such as breast or prostate cancer, derive from normal tissue and often are dependent on sex steroids to maintain growth. Initially, these cancers were treated by surgical ablation of the endocrine glands involved in the secretion of the sex steroids. The surgical removal of the glands that produce an "unwanted" hormone is drastic and far from satisfactory. It was therefore a great advance when hormone antagonist drugs first became available. These hormone antagonists were developed, as were the early hormone agonists, by a laborious process of chemical synthesis of drug candidates and testing in animals. Steroid hormone antagonists with clinical utility include the anti-estrogen tamoxifen, the anti-progestin mifepristone (RU486), and the anti-androgens flutamide and cyproterone acetate. Other clinically useful hormone antagonists include the anti-mineralocorticoid spironolactone.

## Identification of Targets for the Discovery of Novel Drugs

The number of diseases that are associated with inappropriate production of or response to hormonal stimuli highlights the medical and biological importance of these effectors. The recent advances in our understanding of the molecular basis for the action of IRs offer the opportunity to improve many of the existing IR-modulating drugs. Despite the clinical utility of currently available hormone agonists and antagonists, many of the compounds are limited by their side-effect profiles. Delineation of the mechanism of a specific biological response enables the identification of small molecules that retain the efficacy of the IR-modulating drug, but have significantly improved side-effect profiles. Development of compounds more selective for the target IR and, thus, in the function(s) that they elicit are discussed in section I, below.

The discovery of receptor isoforms for a variety of receptor systems has already led to the development of more specific and improved drugs. These include the cardioselective $\beta$-adrenergic blockers and the receptor subtype selective antihistamines. For certain non-protein hormones, identification of intracellular receptor subtypes, many of which are expressed in a tissue specific manner, not only implies a specific physiological role for each subtype but may offer better defined pharmacological effects and thus potential for development of highly selective drugs. The utility of this approach for drug discovery is detailed in section II, below.

Another recent finding, offering additional potential for the discovery of new drugs, is the identification of several dozen "orphan" members of the IR superfamily. In most cases, ligands that activate these so-called orphan receptors have yet to be identified. These IRs may represent as yet uncharacterized signal transduction pathways for novel endocrine and paracrine systems or may include subtypes of IRs for known ligands. In either case the orphan IRs, discussed in section III, below, are of great interest for discovery of more selective and efficacious small molecule drugs.

**I. Tissue-Selective Intracellular Receptor Activators.** Progesterone is produced in the ovaries, testes, adrenal cortex, and placenta. Along with estrogens, progesterone is critical in preparing the female reproductive tract for reception of sperm and implantation of a fertilized ovum. Progestins and estrogens cause growth and development of the reproductive tract and breasts. Progesterone is responsible for the body temperature rise upon ovulation and is also critical for the maintenance of pregnancy.

Therapeutic uses for progestins may include contraception (in combination with estrogens) and control of dysfunctional uterine bleeding, dysmenorrhea (in combination with an estrogen), endometriosis, and threatened spontaneous abortion. The traditional steroidal agonists of the progesterone receptor (*e.g.*, norgestrel and norethindrone) were synthesized over 25 years ago and exhibit anti-estrogenic activity along with varying degrees of cross reactivity with AR and GR.[59] While some cross reactivity with other receptors may occasionally be desirable,[60] opportunities now exist to prepare selective, easily synthesized progesterone receptor agonists and antagonists. Two functionally different approaches by which this may be achieved will now be discussed.

The recent discovery that the human progesterone receptor (hPR) exists in two forms, hPR-A (94 kDa) and hPR-B (120 kDa), opens one avenue for drug discovery. These receptors differ by 164 amino acids, which are present in the N-terminal region of hPR-B but absent from hPR-A. The two receptors have identical DNA and ligand binding domains and may[61] or may not[62] be present in equimolar concentrations in tissues. Hetero- and homodimers of hPR-A and hPR-B form upon ligand activation. Recent data suggest that the cellular pathways used by hPR-A and hPR-B are distinct.[63] The hPR-A has been demonstrated to inhibit the transcriptional activity of the glucocorticoid,[64] estrogen,[64] androgen, and mineralocorticoid[66] receptors in a cell- and promoter-specific manner. These properties may facilitate the identification of more selective compounds or compounds with cross reactivity by allowing *in vitro* analysis of progestin action. For example, hPR-A inhibition of ER may be a mechanism through which the anti-estrogenic properties of progestins are produced. These observations, if they apply *in vivo*, set the stage for discovering a new generation of hPR modulators.

Anti-progestins are currently used acutely as abortifacients. Other possible therapeutic uses may include cervical ripening and treatments for endometriosis, uterine fibroids, meningioma, and breast cancer. The anti-progestins are predominantly 11$\beta$-aryl-19-nor steroids[67], (*e.g.*, mifepristone, onapristone and Org 31806, Figure 5).[68-70] Cross reactivity with the androgen, glucocorticoid, and estrogen receptors is a feature of

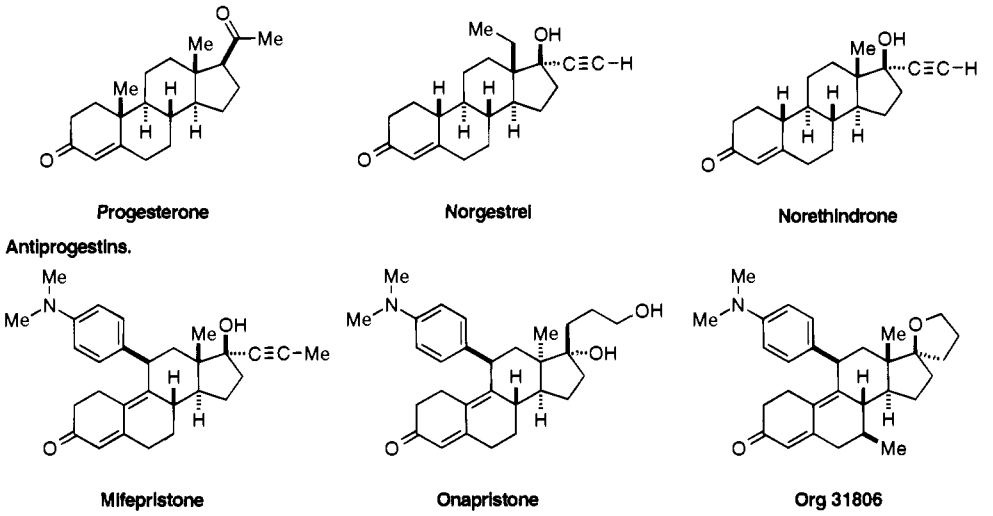


**Figure 5.** Progesterone–receptor agonists and antagonists.

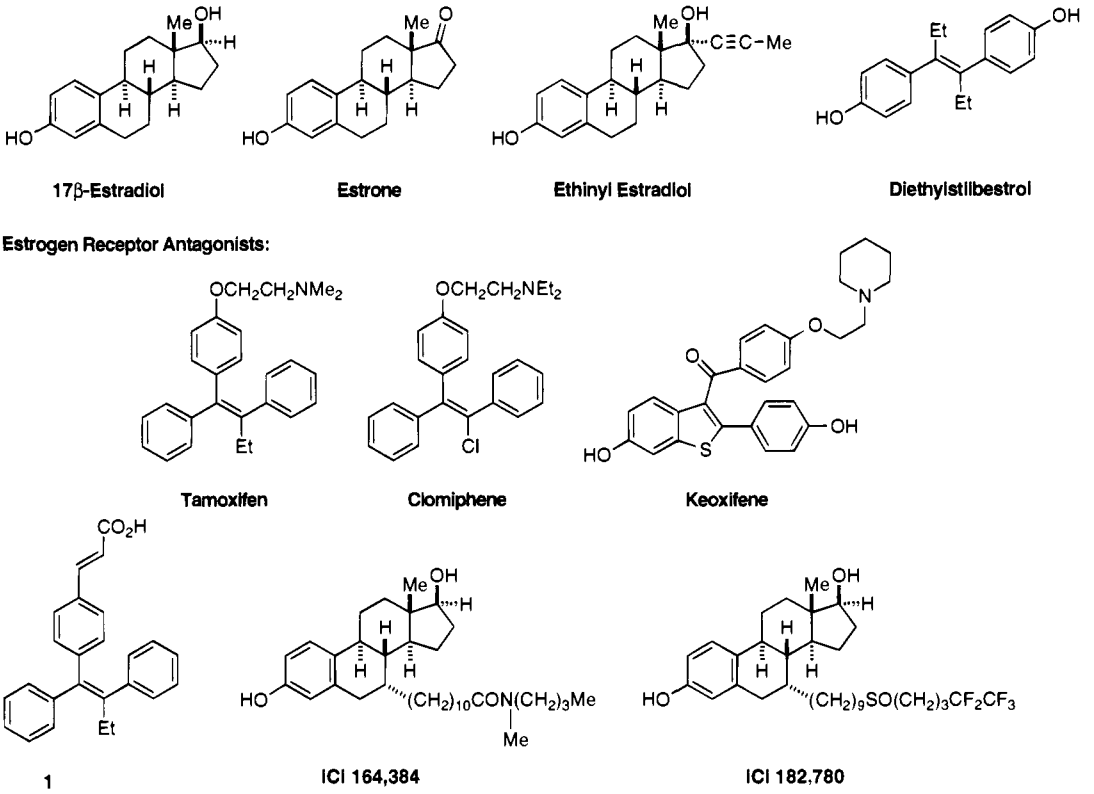


**Figure 6.** Estrogen receptor modulators.

current anti-progestins, which potentially limits their tolerability for chronic administration. Mifepristone[68] is a potent anti-progestin, anti-glucocorticold, and anti-androgen that also exhibits anti-estrogenic behavior. These cross reactivities, while of little consequence for acute uses, may be detrimental in chronic therapy.

Substantial progress has been made to prepare more selective 11$\beta$-aryl steroidal anti-progestins; however, these compounds are difficult to synthesize.[67] Furthermore, some evidence suggests that there are two classes of anti-progestins among the 11$\beta$-aryl steroids[71] that can be differentiated by the affinity of the ligand-bound dimerized receptor complex for DNA. Mifepristone and its analogues induce a dimerized receptor conformation with high affinity for DNA, while onapristone induces a conformation with low affinity for DNA. The clinical relevance of this mechanistic difference remains to be elucidated. Structurally, mifepristone and onapristone differ in stereochemistry at $C_{18}$, with onapristone being inverted from the usual steroid nucleus (Figure 5).

Estrogens are produced primarily in the ovaries and are responsible for stimulation of development of female sex organs, mammary glands, and various secondary sexual characteristics. Therapeutic uses of estrogen agonists include oral contraception (in combination with progestins), hormone replacement therapy in postmenopausal women, and treatment for dysmenorrhea, dysfunctional uterine bleeding, acne, hirsutism, failure of ovarian development, coronary artery disease, osteoporosis, and prostate cancers.

Steroidal estrogens (*e.g.*, 17$\beta$-estradiol, estrone, Figure 6) have a characteristic phenolic A ring. Natural estrogens are deactivated in the liver; however, an $\alpha$-substituent at $C_{17}$ interrupts this metabolism (see

ethinyl estradiol). Several non-steroidal estrogens exist including flavinoids and di- and triphenylethylenes (*e.g.*, diethylstilbestrol).

Currently identified anti-estrogens and partial estrogen agonists are predominantly triphenylethylenes. These include tamoxifen,[72] chlomiphene, keoxifene, and 1 (Figure 6).[73] Steroidal anti-estrogens have been prepared and contain a long side chain at $C_7$ (*e.g.*, ICI 164,384 and ICI 182,780).[74] Breast cancer is the current predominant therapeutic use for estrogen antagonists. Recent molecular insights into the transcriptional transactivating functions of estrogen receptor and other steroid receptors have opened new avenues for drug discovery, making possible the identification of compounds that demonstrate selectivity for a desired biological response.

The estrogen receptor has two distinct regions that confer transactivation of transcription: TAF-1 and TAF-2. Mutant forms of human ER (hER) have been constructed in which either TAF-1 or TAF-2 is genetically "excised". Cotransfection into mammalian cells of TAF-deleted hER genes or wild-type hER cDNAs, together with a plasmid containing an estrogen-responsive reporter gene such as luciferase cDNA, allows the rigorous analysis of the role played by TAF-1 and TAF-2 in the activation of transcription of various target genes by the ER. Using this cotransfection assay, it is possible to dissect the influences on ER-driven transcription of (i) cell background, (ii) promoter context, and (iii) activating ligand (various hER agonists, antagonists, and partial agonists).

When a particular ligand (*e.g.*, estradiol, tamoxifen, or ICI 164,384) interacts with the ER, it induces (or stabilizes) a particular conformation of the receptor. Full agonists, such as 17$\beta$-estradiol, induce a "fully active" conformation, in which both TAF-1 and TAF-2 are "exposed" and active. Full antagonists, such as ICI 164,384, appear to bind to ER (competitively with estradiol) and expose neither TAF-1 nor TAF-2. Partial agonists drive the ER into conformations "intermediate" between the fully active and fully inactive conformations driven by estradiol and ICI 164,384, respectively.

Interestingly, not all partial agonists drive the receptor into the same conformation. Tamoxifen appears to induce "exposure" of TAF-1 but not of TAF-2. Tamoxifen therefore functions as an agonist for TAF-1-dependent functions and as an antagonist for TAF-2-dependent functions. The former appears to underlie its estrogen-mimetic pharmacological effects in uterine tissue,[75] and the latter appears to account for its estrogen-blocking effects in breast cancer. Other compounds drive equally reproducible, partially-activating conformations of the ER, differing from that driven by tamoxifen. With the appropriate partial agonists for the ER, therefore, it is possible to achieve transcriptional enhancement of only a subset of estrogen-responsive genes. Compounds with a subset of estrogen's full spectrum of activities can be identified using assays based upon these principles.

Once exposed as a consequence of a ligand-induced conformational change in the ER, TAF-1 and TAF-2 can each independently enhance transcription of some estrogen-responsible genes, presumably by interacting with specific intracellular partner proteins. The recent identification of a 160 kDa estrogen receptor-associated protein (ERAP160)[76] lends further credence to this model. ERAP160 binds to ER through interactions

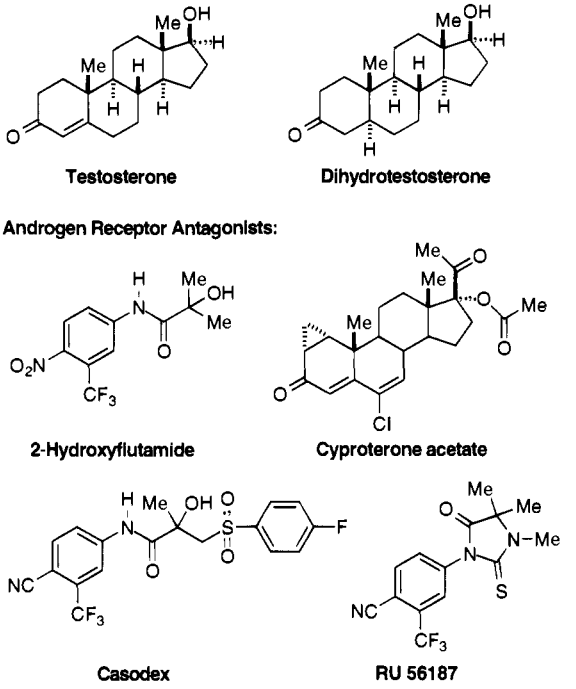


**Figure 7.** Androgen receptor agonists and antagonists.

involving TAF-2 in an estradiol-dependent manner. The binding of anti-estrogens to the receptor blocks the binding of ERAP160. It has been suggested that ERAP160 mediates transactivation of ER and that the ability of anti-estrogens to block ER-ERAP160 complex formation may account for their therapeutic effects in breast cancer. Although the identities of other postulated partner proteins remain unknown, indirect evidence indicates that their expression varies from cell type to cell type. The identification of these postulated partner proteins will provide yet another attractive target for the development of highly selective small-molecule drugs.

Emerging evidence supports the utility of an approach similar to that taken with ER and PR for the development of tissue-selective partial agonists for other receptors including PR, AR, MR, GR, and VDR.

Androgens are synthesized in the testes, adrenal cortex, and ovaries. The net effect of endogenous androgens reflects the combined actions of the secreted hormone, testosterone (Figure 7); its 5$\alpha$-reduced metabolite, dihydrotestosterone; and its estrogenic derivative, estradiol. Androgens serve different functions at different stages of male development and have clear therapeutic uses in the treatment of hypogonadism, growth retardation, breast carcinoma, and osteoporosis. The actions of androgens are mediated through AR.[21-23] Compounds that block the action or synthesis of androgens have proven useful in treatment of diseases such as prostate cancer, prostatic hypertrophy, hirutism, male pattern baldness, and acne. Among the most potent orally active anti-androgens (Figure 7) is cyproterone acetate. This compound possesses progestational activity and suppresses the secretion of gonadotrophins, both of which are unwanted side effects. Other anti-androgens include flutamide, a prodrug for the active metabolite, 2-hydroxyflutamide,[77] casodex,[78] and an analogue of nilutamide.[79]

Glucocorticoids and mineralocorticoids are steroid hormones produced by the adrenal cortex. Glucocorti-

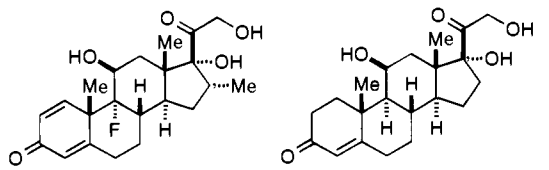


**Figure 8.** Glucocorticoid receptor agonists.

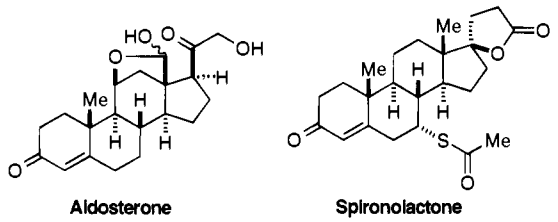


**Figure 9.** Mineralocorticoid receptor modulators.

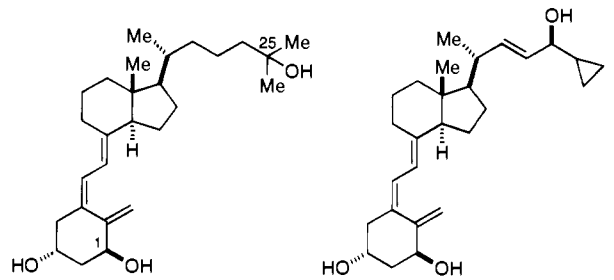


**Figure 10.** Vitamin D receptor agonists.

coids influence almost every organ and tissue in the body, affecting responses as diverse as behavior, immune function, and carbohydrate metabolism. Cortisol is the most potent naturally occurring glucocorticoid and stimulates or inhibits various biological functions. Synthetic glucocorticoid agonists can be divided into two groups: (i) 4-pregnene or 4-androstenc derivatives and (ii) derivatives of cortisol or dexamethasone (Figure 8). It is not surprising, when the many actions of the glucocorticoids are considered, that these compounds actually have mixed agonist–antagonist activities when examined *in vivo*.

Mineralocorticoids (Figure 9) such as aldosterone regulate electrolyte balance in the kidneys, salivary glands, sweat glands, and gastrointestinal tract. Aldosterone acts by altering the ATP-dependent membrane transport of sodium and potassium ions. The action of aldosterone is inhibited by spironolactone and progesterone. Both act as competitive inhibitors of aldosterone by forming ligand–receptor complexes that are inactive.

A vitamin $D_3$ precursor is photochemically synthesized in the skin from 7-dehydrocholesterol and then undergoes a hydrogen shift to become vitamin $D_3$. As with hormones secreted by endocrine glands, this product is transported in the blood to distal sites where it is metabolized to the hormonally active form, calcitriol (1α,25-dihydroxyvitamin $D_3$, Figure 10), which then affects target tissues through interaction with the VDR, eventually resulting in increased plasma calcium concentrations. The conversion of vitamin $D_3$ to calcitriol occurring in the kidney is regulated by a negative-feedback control involving free calcium concentrations in the plasma.

The role of calcitriol in controlling the expression of a broad spectrum of genes is becoming increasingly evident. In addition to its active role in calcium homeostasis, calcitriol regulates genes associated with cell growth and tissue-specific structure. It is also responsible for maintaining the precise control of the concentration of calcium and phosphate ions in the plasma by modulating their absorption from the small intestine, enhancing their mobilization from bone and altering their excretion via the kidney. A growing body of evidence indicates that calcitriol also plays a role in the control of proliferation and differentiation of several cell types including epidermal keratinocytes. This effect of VDR agonists on skin has been utilized clinically in the treatment of psoriasis. Vitamin D analogues are also capable of causing differentiation of malignant cells, driving interest in VDR agonists in the treatment of leukemias and breast cancer.

All of the genomic effects of calcitriol are mediated by the VDR, which has been characterized biochemically from a number of tissues derived from many different animal species. There is evidence that certain rapid effects of vitamin D may reflect direct non-VDR-mediated actions at the plasma membrane. One compound that displays tissue-selective VDR agonist action is MC 903 (Figure 10),[80] which mimics the effects of vitamin $D_3$ on skin without increasing plasma calcium concentrations. It is not presently clear to what extent the tissue-selective actions of compounds such as MC 903 reflect intrinsic pharmacodynamics or pharmacokinetics or differential drug distribution. There is potential for other such tissue-specific vitamin $D_3$ partial mimics in the treatment of various skin diseases and cancers.

**II. Intracellular Receptor Subtype Selective Compounds.** Vitamin A (retinol) is derived exclusively from the diet as preformed retinol, retinyl esters, or carotenoids (provitamin A) and is stored primarily in liver as retinyl esters. Like vitamin $D_3$, retinol is transformed in the body to a variety of active metabolites that play important roles in several diverse cellular processes, including embryonic development, vision, reproduction, bone formation, hematopoiesis, metabolism, cellular differentiation, cellular proliferation, and programmed cell death.[81]

Retinal (vitamin A aldehyde) is required for retinal function. Other vitamin A derivatives, including *all-trans*-retinoic acid (ATRA or vitamin A acid), play an essential role in growth and differentiation of epithelial tissue and are necessary for reproduction, embryonic development, and bone growth. These actions of ATRA and related retinoic acid isomers (i.e., 9-*cis*-RA, discussed below) are mediated by RA IRs, which regulate gene expression.[82]

The profound effect s of retinol metabolites on cellular differentiation and proliferation have spurred the synthesis of thousands of RA analogues (retinoids) with potential use in a variety of skin disorders and malignant disease. Presently, the naturally occurring retinoids (Figure 11) ATRA (an active hormone) and 13-*cis*-RA (most likely acting by giving rise to ATRA and possibly 9-*cis*-Ra) are used for the treatment of severe acne, while synthetic etretinate is prescribed for severe, refractory psoriasis. More recently, ATRA and 13-*cis*-RA have shown promise in the control of cancers or precancers such as acute promyelocytic leukemia,[83] head

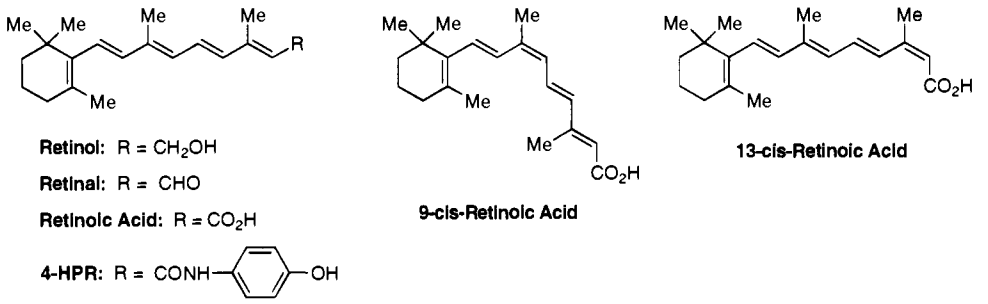


**Figure 11.** Retinoic acid and derivatives.

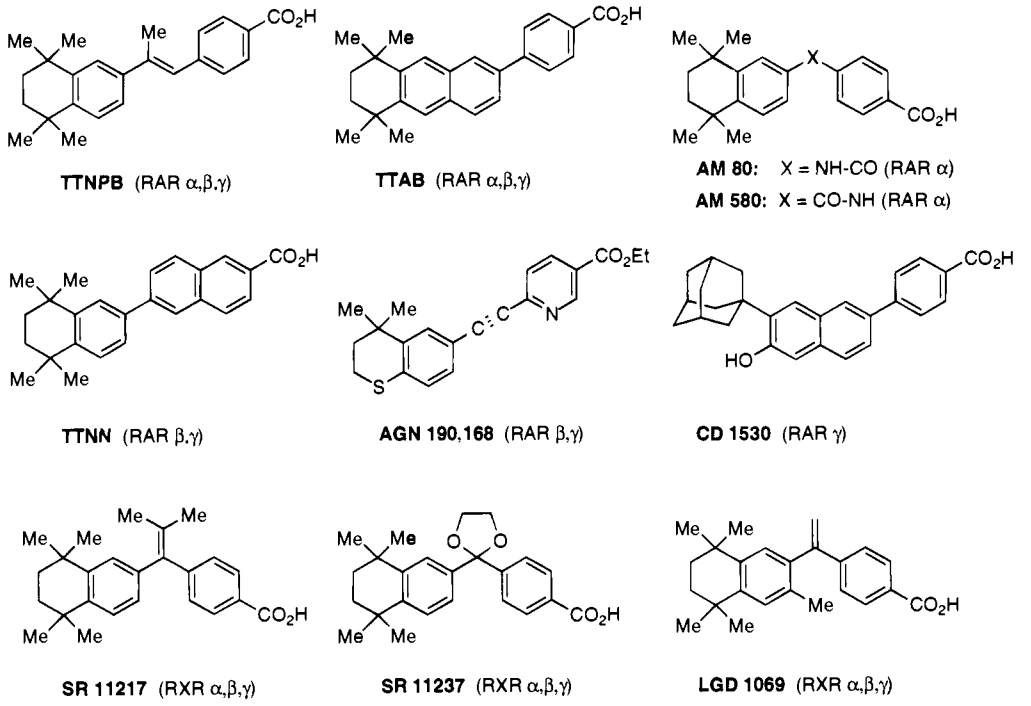


**Figure 12.** Synthetic retinoids.

and neck cancer,[84] and cervical dysplasia,[85] either as single agents or in combination with other agents such as interferon α. Other synthetic retinoids are in various stages of development for treatment of cancer and skin diseases, including *N*-(4-hydroxyphenyl)retinamide (4-HPR), which is in phase II trials as a chemopreventive agent in breast cancer treatment.[86] Retinamide is likely a prodrug for ATRA.

Unfortunately, widespread clinical use of the currently available retinoids is limited by undesirable side effects. These include mucocutaneous irritation, elevations in plasma triglycerides, headache, bone toxicity, and teratogenicity. The many diverse actions of retinoids, both desirable and undesirable, arise through activation of multiple retinoid receptor subtypes; thus retinoids with receptor subtype selectivity may have improved therapeutic indices.

To date six IR subtypes (or isoforms) that can be activated by ATRA in cells have been identified.[82] Each receptor is encoded by a separate gene. Three of these, RARα, RARβ, and RARγ, are close genetic homologues. ATRA binds directly to each of the RARs, leading to activation of the RARs as transcription factors. The remaining three IRs responsive in cell culture to ATRA are members of the retinoid X receptor subfamily and are designated RXRα, RXRβ, and RXRγ. The RXRs are close genetic homologues of each other, but are less closely related to the RARs. Although the RXRs can be activated by ATRA in living cells, ATRA does not bind to the RXRs directly. ATRA activates RXRs indirectly upon conversion to 9-*cis* RA, the endogenous ligand for the RXRs, which binds to and activates both RXRs and RARs.[87] The pharmacological effects of 9-*cis*-RA, the first novel non-peptidyl hormone described since vitamin $D_3$ was discovered in 1968, imply possible utility in the treatment of cancer and skin diseases. Chemically synthesized 9-*cis*-RA (LGD1057) is currently in clinical trials in oral and topical formulations for cancer indications.

In addition to endogenous retinoids such as ATRA, 9-*cis*-retinoic acid and 13-*cis*-RA, synthetic, non-natural retinoids with novel retinoid receptor subtype selectivity are emerging as potentially exciting drugs. Investigators have used receptor binding and cotransfection assays to characterize known synthetic retinoids and newer analogues (Figure 12). Highly potent retinoids such as TTNPB and TTAB selectively activate the RAR subfamily but do not effectively distinguish among the isoforms.[88] Both AM-80 and AM-580 display selectivity for the RARα isoform,[89] while TTNN is representative of compounds selective for the RARβ and RARγ subtypes.[88,89] Phase III clinical trials for topical treatment of acne and psoriasis have been completed using AGN 190,168, a novel RARβ- and RARγ-selective compound.[90]

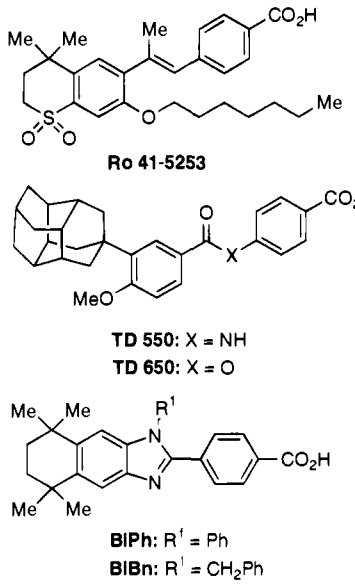


**Figure 13.** RAR antagonists.

CD 1530 is representative of structures reported to selectively activate the RAR$\gamma$ subtype.[91]

Within the past 2 years, novel classes of RXR-selective retinoids have also been described. Both SR11217 and SR11237 display activation profiles for RXR$\alpha$ similar to that of 9-*cis*-RA at 100 nM.[92] Neither compound activates RAR subtypes. Recently, a potent series of RXR-selective retinoids was developed by capitalizing on the observation that incorporation of a methyl substituent at the 3-position of the tetrahydronaphthalene moiety of TTNPB results in a retinoid with modest activity at all RAR and RXR subtypes.[93] A potent member of this new class, LGD1069, represents the first RXR-selective synthetic retinoid to enter clinical trials for the treatment of cancer.

Several RAR-selective antagonists have also recently been described. A series of sulfone derivatives related to Ro 41-5253 exhibits selectivity for the RAR$\alpha$ subtype (Figure 13).[94] Ro 41-5253 was shown to antagonize the teratogenic effects of the RAR$\alpha$-selective agonist AM-580 in rat limb bud cell cultures and in mice. Additional RAR antagonists include TD550, TD650, BIPh, and BIBn, which inhibit retinoid-induced differentiation of human promyelocytic leukemia HL60 cells.[95,96] As yet, no RXR-selective antagonists have been reported.

Data now emerging support separable biological roles for the various RAR and RXR subfamilies and individual subtypes in the control of cell proliferation, differentiation, and programmed cell death (apoptosis). RAR-selective compounds are sufficient to stimulate replication of human cytomegalovirus (hCMV) and induce the differentiation of an embryonal cell line that supports the growth of hCMV.[97] Further analysis of the pharmacological actions of the retinoids and identification of more selective analogues, in conjunction with studies using molecular and cellular biological approaches, are driving elucidation of the biological roles of the retinoid receptor subtypes and the delineation of the potential therapeutical uses of receptor subtype selective retinoids. Additional synthetic retinoids with useful patterns of selective interaction with the RAR and RXR subtypes hold great promise as pharmacological tools for biological investigations and as novel pharmaceuticals.

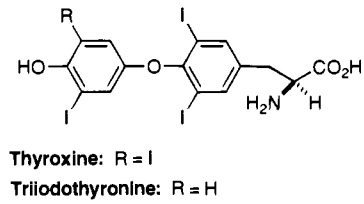


**Figure 14.** Thyroid hormones.

The thyroid gland is the source of two different thyroid hormones. Thyroxine ($T_4$) and triiodothyronine ($T_3$)[98] (Figure 14) are essential for normal growth and development and play an important role in controlling energy metabolism. Changes in the cardiovascular system are prominent consequences of the action of thyroid hormones. Stimulation of cholesterol metabolism to bile acids and lower plasma cholesterol levels result from elevated levels of thyroid hormones. A great many structural analogues of thyroxine have been synthesized in order to define structure–activity relationships, detect antagonists of thyroid hormones, or find compounds exhibiting desirable activity with reduced unwanted side effects.

Three TR subtypes, TR$\alpha$1, TR$\alpha$2, and TR$\beta$1, have been identified in human tissues.[99] A fourth receptor subtype, TR$\beta$2, was subsequently isolated from the rat[100] and has been identified in human tissues.[101] The expression of the different TR subtypes is regulated both transcriptionally and post-transcriptionally. Each messenger RNA encoding a TR subtype shows characteristic patterns of developmental, tissue-specific, and hormonal regulation. The complexity of the TRs and their differing patterns of expression suggest that some of the myriad actions of thyroid hormone are mediated by specific TR subtypes. Such specific physiological roles of TR subtypes would imply better defined pharmacological effects for thyroid hormone agonists with TR subtype selectivity.

For other hormones of medical importance, including glucocorticoids and estrogens, receptor subtypes have not yet been identified, although in some senses MR can be thought of as a GR subtype at sites outside the kidney. Among the many orphan receptors (see below) and novel IRs that continue to be identified, currently unrecognized IR subtypes for known hormones may await identification.

**III. Orphan Receptors.** The ongoing discovery of orphan IRs continues to expand the list of superfamily members. The various orphan receptors are likely to play important functional roles, since (i) their sequences are highly conserved in mammals and even between phyla, (ii) they often have restricted spatial and temporal patterns of expression, (iii) transgenic animals in which various orphan receptors have been "knocked out" show functional impairment or lethality, and (iv) at least some of the orphan IRs can be implicated in the control of specific promoters (for example, hepatic nuclear factor-4 [HNF-4]). For the most part, the functional roles of these orphan IRs *in vivo* remain unknown. It is likely that some of these orphan receptors represent the signal-transducing receptors for currently uncharacterized endocrine, paracrine, or intracrine hormonal regulatory systems. Other orphans IRs may represent subtypes of receptors for known ligands. Thus the RXRs (originally considered orphans) bind and are activated by 9-*cis*-RA.[87]

The restricted tissue distribution of some orphan IRs and the responses of these orphan receptors to known pharmacophores invite speculation about their possible functions. For example, orphan IRs, including chicken ovalbumin upstream promoter transcription factor (COUP-TF),[102] COUP-TF$\beta$,[103] and HNF-4,[104] and the three peroxisome proliferator activated receptor (PPAR) subtypes (PPAR$\alpha$, PPAR$\beta$ or NUC1, PPAR$\gamma$),[105] appear to be involved in the control of lipid, cholesterol, or lipoprotein metabolism, rendering them interesting from a pharmaceutical perspective. Known xenobiotics and drugs, such as the fibrate antihyperlipidemics, which induce peroxisome proliferation, appear to act through PPARs. Other orphan receptors, *e.g.*, nerve growth factor induced-B (NGFI-B)[106,107] and the related Nur77 are expressed after cellular exposure to NGF or other growth factors and appear to influence cellular susceptibility to apoptosis. Although their function remains to be elucidated, the thyroid-related (TR2) orphan IR[101] and its splice variants are expressed in a pattern essentially limited to the tissues of the genitourinary tract. Orphan IRs and other members of the superfamily also exhibit overlapping tissue and developmental distribution together with overlapping specificity for response elements within target genes. In these instances expression of target genes in a given tissue mauy be determined by interaction between members of the superfamily receptor complement present in that tissue.

Additional complexity is added with the identification of a growing number of IR subfamily members, as exemplified by the retinoid receptor family. The RXR subfamily was the first retinoid-related subfamily to be identified. Members of this subfamily form heterodimers not only with the retinoid-related RARs, but also with other hormone-activated and orphan IRs including VDR, TR, and PPARs. Recently, the RAR-related orphan receptors (RORs)[108] and retinoid Z-related receptors (RZRs)[109] were identified. The RORs share common DNA- and putative ligand-binding domains, but differ in the N-terminal domains that are generated by alternative RNA splicing. Different members of this subfamily show different binding affinity for the RAR-related orphan response element (RORE) and, as a result, are able to mediate both constitutive and low-level transcription activation of target genes. RZR$\alpha$ and -$\beta$, although sharing a high degree of homology, have a different tissue distribution, with expression of RZR$\beta$ confined to brain tissue. The RZRs bind as monomers to natural and artificial retinoic acid response elements containing hexameric half-sites and are also able to form homodimers on selected response elements.

Lastly, there are orphan IRs that appear to be constitutively active in the absence of added ligands. These receptors may actually be responding to "intracrine" small-molecule ligands such as metabolic intermediates, *e.g.*, certain fatty acids to which PPARs respond. It is not necessarily the case that all orphan IRs have activating endogenous ligands. For example receptors such as COUP-TF and PR (in some species) can be activated by phosphorylation of appropriate residues by protein kinase A;[110] the physiological and pharmacological relevance of such phosphorylation in modulation of IR activity remains to be definitively established. In any event the orphan IRs potentially are novel targets for pharmaceutical intervention. The actualization of the potential inherent in the orphan IRs is a major challenge in IR-related drug discovery.

**STATs and Drug Discovery.** In addition to the steroid and small molecule hormones that control gene expression through interactions with IRs, there are peptide and protein ligands in the systemic circulation that produce alterations in gene expression in their target cells to which they bind. Included in this class of proteins are the cytokines (*e.g.*, the interferons and interleukins) and growth factors (*e.g.*, epidermal growth factor). The modulatory proteins are collectively termed extracellular signaling proteins (ESPs).[4] ESPs cannot readily enter cells; they act by binding at the cell surface to specific receptors that span the cell membrane. Thus they can indirectly initiate a chain of events (characterized only recently for a growing number of ESPs) that culminate in changes in the pattern of cellular gene expression. The exact changes elicited are characteristic for the inciting ESP stimulus. For example, after a cell is exposed to interferon-$\alpha$, specific genes are expressed yielding proteins that (i) render the cell more resistant to viral infection and (ii) reduce the rate at which the cell proliferates. These specific changes in gene expression following exposure of cells to ESPs are in many cases mediated by members of a newly discovered transcription factor superfamily called STATs.[4]

The STATs characterized to date range in molecular mass from roughly 80 to 113 kDa and are not genetic homologues of any other known group of proteins. Among the ESPs, which at least in part appear to exert their effects on cells through specific STATs,[111] are the interferons (IFNs) [IFN$\alpha$, -$\beta$, and -$\gamma$]; the colony-stimulating factors (CSFs) [erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF), and probably the recently described thrombopoietin (Tpo)]; various interleukins (ILs) [IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13]; various growth factors [including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), oncostatin-M (OncoM), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF)]; and several peptidyl hormones [*e.g.*, growth hormone (GH) and prolactin].

The identification of the first STATs[4,112] led to the elucidation of the biochemical events that mediate the changes in gene expression in response to interferons. Surprisingly, there is an underlying unity in the way that many additional ESPs, with their myriad of distinctive biological effects, act to control gene expression after binding to their cell surface receptors. In response to an ESP stimulus, specific STAT proteins are phosphorylated on tyrosyl residues within minutes after the binding of the ESP to its cell-surface receptor. This phosphorylation of STAT proteins is mediated by specific kinases called Janus kinases, or JAKs (see below) and results in their conversion from latent to active transcription factors.

To date, six different STAT family members (STATs1, -2, -3, -4, -5, -6) have been discovered, and as shown in Table 2 the cytokines that activate all of these STATs have been identified. However, it is highly likely that additional STATs will be found that participate in signal transduction of still other cytokines.

**Table 2.** Cytokines Utilizing the JAK/STAT Pathway

| cytokine | STAT activation | JAK activation[a] |
|---|---|---|
| IFNα | STAT1, −2 | JAK1, Tyk2 |
| IFNγ | STAT1 | JAK1, JAK2 |
| IL-10 | STAT1, −3 | JAK1, Tyk2 |
| IL-2 | STAT5, −3 | JAK1, −3 |
| IL-7 | STAT5, −3 | JAK1, −3 |
| IL-9 | STAT5, −3 | JAK1, −3 |
| IL-15 | STAT5, −3 | JAK1, −3 |
| IL-4 | STAT6 | JAK1, −3 |
| IL-13 | STAT6 | JAK1 |
| IL-3 | STAT5 | JAK2 |
| IL-5 | STAT1, −3 | JAK2 |
| GM-CSF | STAT5 | JAK2 |
| IL-6 | STAT1, −3 | JAK1, −2, Tyk2 |
| IL-11 | STAT3 | JAK1, −2, Tyk2 |
| LIF | STAT3 | JAK1, −2, Tyk2 |
| OSM | STAT1, −3 | JAK1, −2, Tyk2 |
| CNTF | STAT3 | JAK1, −2, Tyk2 |
| IL-12 | STAT3, −4 | Tyk2, JAK2 |
| G-CSF | STAT3, −5 | JAK1, −2 |
| Epo | STAT5 | JAK2 |
| Tpo | STAT5 | JAK2 |
| prolactin | STAT5 | JAK2 |
| growth hormone | STAT1, −3, −5 | JAK2 |
| CSF-1/M-CSF | STAT1, −3 | ? |
| EGF | STAT1, −3, −5 | JAK1 |
| PDGF | STAT1, −3 | ? |

[a] A question mark (?) indicates that JAK activation has not been reported.

The colony-stimulating factors and interleukin-3 promote the growth of specific cell lineages within the bone marrow, giving rise to the mature cell types found in the blood. GM-CSF and G-CSF are currently used following cancer chemotherapy to increase the speed with which the white blood cell counts return to levels that are protective against infection or to facilitate the process of bone marrow transplantation in cancer patients. Epo has been extraordinarily successful in treatment of anemia due to renal failure. Interferon-β is used in the management of relapsing multiple sclerosis. Interferon-α is one of the cytokines produced by cells in response to viral infection. Recombinant IFN-α has been successfully utilized in the treatment of infectious hepatitis, hairy cell leukemia, and other cancers. The clinical utility of a variety of other cytokines is currently being assessed. The definition of the mechanisms by which the JAKs and STATs are activated and an understanding of their role in ESP signal transduction present new opportunities to discover orally bioavailable small molecule drugs mimicking or blocking medically important ESPs.

## Mechanism of JAK/STAT-Mediated Signal Transduction

After an ESP binds to its cognate receptor, a cascade of events is initiated that leads to modulation of gene expression. The primary driver of this cascade appears to be protein phosphorylation. Receptor occupancy, probably through receptor dimerization, leads to changes in the cytoplasmic domain of the receptor that are "recognized" intracellularly. Evidence indicates that the altered receptor cytoplasmic domain becomes an effective "docking platform" for the appropriate members of a tyrosine protein kinase family known as the "Janus kinase" or JAK family[113−115] and/or members of the STAT family of latent transcription factor subunits. Assembly of an appropriate complex of JAKs and STATs anchored to the cytoplasmic domain of various ESP receptors results in tyrosyl phosphorylation and biochemical activation of particular JAKs. The activated JAKs then phosphorylate a subset of STAT proteins at a specific tyrosyl residue.

Although some receptors that utilize the JAK/STAT signaling pathway have intrinsic tyrosyl kinase activity (*e.g.*, receptors for EGF and PDGF), most of these receptors do not. Furthermore all STAT phosphorylation is thought to be dependent upon the JAK kinases that associate noncovalently with the cytoplasmic domains of various ESP receptors. The receptors for ESPs are diverse and, with the exception of those for tumor necrosis factor (TNF) and IL-1, all seem to couple to the STAT signaling pathway through JAK activation. Receptors that bind ESPs as single chains (*e.g.*, receptors for growth hormone, Epo, prolactin, and G-CSF) dimerize after ESP binding. This dimerization appears to lead to localization and activation of particular JAKs. Receptor multimerization also appears to be involved in JAK activation by ESPs that bind receptors with multiple chains. The receptors for one group of cytokines (IL-3, GM-CSF, and IL-5) are formed from different α chains and a common β chain. JAKs interact with the β chain. Lastly, there are cytokines (IL-6, LIF, OncoM, and CNTF) whose receptors are composed of specific α chains and a common protein component termed gp130. The action of these ESPs depends upon oligomerization of the gp130 subunit, which associates with specific JAKs, to activate the STAT signaling pathway.

Tyrosyl-phosphorylated STATs assemble into multimeric complexes, apparently stabilized by intermolecular interactions between src homology 2 (SH2) domains[112,116] and phosphorylated tyrosyl residues within the STATs. SH2 domains, originally defined based on the src oncogene, are involved in binding to phosphotyrosine residues. A central region in the SH2 domain that includes the arginine residue that directly binds to phosphotyrosine is completely conserved among the STAT proteins. Different specific STAT complexes appear to be induced in response to different ESPs. These STAT-containing complexes move from the cytoplasm into the nucleus. Once in the nucleus, activated STAT complexes bind to specific response elements in the promoters of genes responsive to that ESP, acting as biochemically active transcription factors. Following ESP activation of JAK/STAT signaling, the cascade of events appears to be negatively regulated by the action of protein tyrosyl phosphate phosphatases. An important role for protein tyrosyl phosphate phosphatases in limiting ESP-mediated responses is implied by the ability of the protein phosphatases inhibitor vanadate to activate JAK/STAT-mediated transcription.[117]

JAK/STAT-mediated changes in the pattern of gene expression lead to alterations in levels of corresponding encoded proteins and therefore to altered cell function. As with hormone-induced IR-mediated changes in gene expression, the consequences of STAT signal transduction show a gradual onset (minutes to hours) and can last a relatively long time (hours to days). The STATs therefore exert their activity in a manner analogous to that of the IRs: the STATs transduce ESP signals that

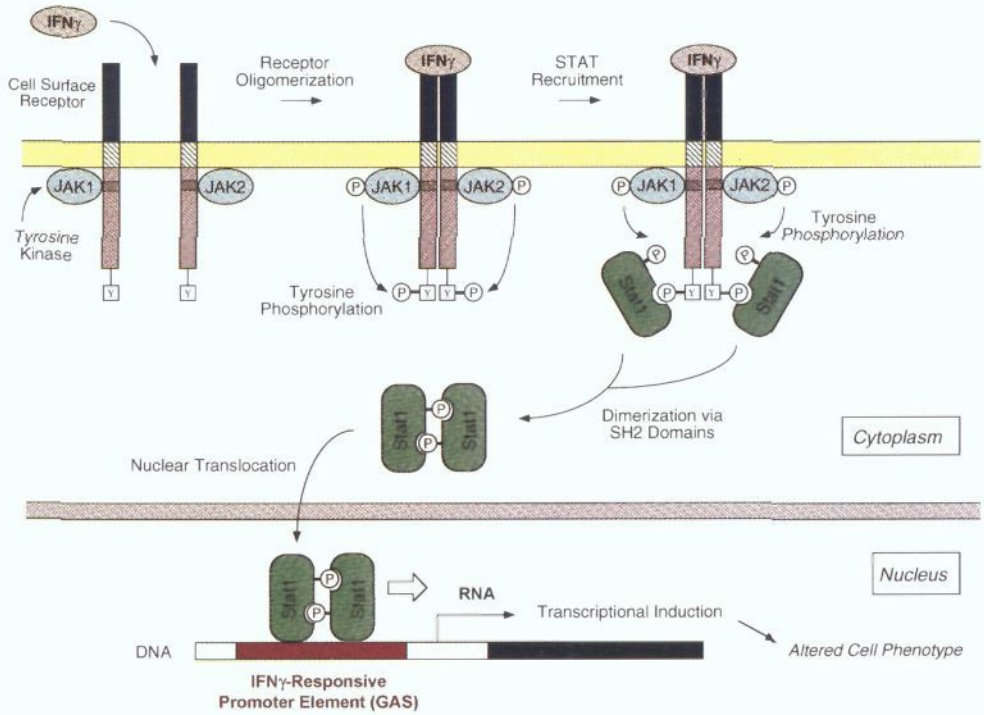


**Figure 15.** The signal transduction pathway utilized by interferon-$\gamma$ (IFN-$\gamma$). Binding of IFN-$\gamma$ to the IFN-$\gamma$ receptor causes multimerization of the receptor and activation of JAK1 and JAK2. These activated JAKs phosphorylate STAT1. This leads to formation of the active transcription factor. After entering the nucleus, the STAT1 homodimer recognizes the IFN-$\gamma$ activation sequence (GAS) in the promoters of IFN responsive genes and enhances transcription of those genes.

control gene expression, and the IRs serve a similar role for the non-peptidyl hormones.

The signal transduction pathway utilized by IFN-$\gamma$ is shown in Figure 15. As described above, the first step in IFN-$\gamma$ signal transduction is the binding of IFN-$\gamma$ to its cell surface receptor. Receptor binding results in receptor oligomerization (a simplified depiction is shown in Figure 15), leading to activation of the receptor-associated JAKs, presumably by cross-phosphorylation. The receptor cytoplasmic domain is then in turn phosphorylated on tyrosine, presumably by the JAKs themselves. A single phosphorylated tyrosine residue on the receptor serves as a docking site for STAT1 (via its SH2 domain); STAT1 then becomes phosphorylated on a specific tyrosyl residue, again presumably via the JAKs.[118] The phosphorylation of this STAT leads to its dimerization, yielding an active transcription factor. The STAT1 dimer can then move into the nucleus and bind to a DNA sequence element, known as an IFN-$\gamma$ activation sequence (GAS), in the promoter of IFN-$\gamma$ responsive genes. Binding of the STAT1 dimer to the promoter of these genes causes transcriptional activation in a manner analagous to the IRs. Although IFN-$\alpha$-induced signal transduction is very similar to that of IFN-$\gamma$, the STATs activated are different as is the makeup of the DNA binding complex. Thus, IFN-$\alpha$ treatment leads to activation of JAK1 and tyk2 and phosphorylation of STAT1 and STAT2. Unlike IFN-$\gamma$ and most other cytokines, the IFN-$\alpha$-induced multimeric complex, termed interferon-stimulated gene factor-3 (ISGF3)[4,119] also includes a DNA-binding protein, termed p48, that is not a STAT family member. In the absence of the activated STAT components, p48 shows only weak DNA binding. The ISGF3−STAT complex moves into the nucleus, specifically recognizes IFN-$\alpha$ stimulated response elements (ISREs) within the promoters of genes responsive to IFN-$\alpha$, and enhances the transcription of those genes.

The STATs and JAKs implicated in signal transduction of a variety of ESPs are shown in Table 2. Although there is significant overlap in the STATs participating in complexes induced by different ESPs, it is believed that selective action of a single ESP is obtained based upon the STATs and accessory proteins such as p48 forming the active complex, the overall transcription factor pool within the specific cell type, the precise nature of the STAT response element,[120] and the promoter context in which the STAT response element resides.

## Discovery of Drugs Modulating ESP Action

Administration of specific ESPs (*e.g.*, Epo, G-CSF, GM-CSF), various interferons, or IL-2 can have a medically beneficial effect. The therapeutically useful ESPs are often relatively difficult and expensive to manufacture, and they must be administered parenterally and frequently. In some pathological conditions, it may be highly desirable to specifically inhibit the actions of individual ESPs. Currently there are no small-molecule drugs known to act by directly modulating ESP-induced JAK/STAT-mediated signal transduction. However, the known physiological and pharmacological activities of many of the ESPs and their putative roles in the pathophysiology of various diseases suggest possible utilities for small-molecule ESP antagonists. Examples include possible use of interleukin or interferon antagonists in the treatment of inflammation or CSF inhibitors in treatment of leukemias.

A variety of assays can be considered in the construction of screens for compounds capable of modulating ESP-signaling pathways. Among these is a cellular

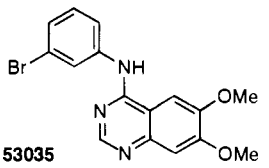


**Figure 16.** Inhibitor of epidermal growth factor receptor tyrosine kinase.

transcriptional assay similar to that used for intracellular drug discovery. In this approach a cellular background that harbors the required components of the signaling pathway could be used (*e.g.*, ESP receptor, JAKs, STATs, and tyrosine kinase phosphatases); alternatively, plasmid constructs capable of expressing one or more of the components of the signaling pathway (*e.g.*, ESP receptors, JAKs, or STAT components) can be introduced into the cell. A plasmid consisting of a promoter containing a STAT response element controlling transcription of a reporter gene such as luciferase is then introduced into the cell. Alteration in reporter gene transcription is then used to monitor the efficacy and potency of compounds tested in the screen.

Second, enzymatic assays for selected kinases and phosphatases can be established to identify compounds[121] capable of directly affecting the activity of these critical components of the signaling pathway. In this regard, a very potent and selective inhibitor of the intrinsic tyrosine kinase activity of the EGF receptor has been identified[122] (Figure 16), suggesting that sufficient structural heterogeneity may exist among tyrosine kinases to allow the discovery of selective and potent kinase inhibitors.

Finally, assays can be established that assess physical interactions of the components of the signaling pathway. These could include the interaction between the ESP receptor and the appropriate JAKs, the receptor and STATs, JAKs, and STATs, and homodimeric or heterodimeric STAT complexes.

Each of these approaches has potential advantages and disadvantages, but all could be used to identify novel small molecules capable of modulating the signal transduction pathway induced by selected ESPs. Drug discovery opportunities in this area are rapidly emerging. Small-molecule agonists could act in a variety of steps in the signaling pathway, including inhibition of phosphatase activity or stabilization of interactions between the various components of the pathway. The evidence cited above demonstrating that vanadate induces transcriptional activation of an ESP response element in the absence of the ESP suggests that JAK/STAT pathways are kept quiescent by the continuous action of protein tyrosyl phosphate phosphatases. Any compound that perturbs the activity or localization of these phosphatases would be expected to activate the pathway. In addition, nuclear protein tyrosyl phosphate phosphatases may be involved in the inactivation of phosphorylated STATs. Compounds that act by stabilizing protein–protein interactions, such as the immune suppressants cyclosporin and FK506 are precedents for the sorts of compounds that might stabilize STAT complexes.

Small-molecule ESP agonists could be therapeutically valuable in a variety of important disease states, for example by replacing (i) Epo for treatment of anemia; (ii) G-CSF, GM-CSF or Tpo as an adjunct to cytotoxic cancer chemotherapy; (iii) IFN-α for induction of an antiviral state to treat infectious hepatitis; (iv) IL-2 or IFN-α for cancer therapy; and (v) growth hormone.

ESP antagonists could also act at several different points in the STAT-signaling pathway. These include binding to the cell-surface receptor, disrupting physical interactions that lead to receptor dimerization, preventing interactions between JAK and STAT molecules, interfering with interaction between JAK/STAT complexes and the general transcription complex, and specifically inhibiting JAK activity.

Antagonists of ESP signaling could be useful in inflammatory disease exacerbated by a variety of ESPs including the interferons IL-2, IL-4, and Il-6. IL-2 antagonists could be used in immunosuppressive therapy for graft rejection while an IL-4 antagonist could reduce allergic symptoms. EGF or PDGF antagonists have potential utility in treatment of growth factor dependent cancers. Thus, multiple drug discovery opportunities are represented by modulation of ESP action through the JAK/STAT signaling pathway.

## Future Aspects

The progress made in understanding the mechanisms through which extracellular signals, such as hormones and cytokines, act to effect gene transcription has provided many new and interesting avenues for development of therapeutically important small-molecule drugs. As these signal transduction pathways are dissected further, the importance of receptor conformation, receptor interactions with accessory proteins, the roles of different subclasses of receptors, interactions between different IRs, and the role of orphan receptors in determining the specificity of the action of extracellular signals will become clear. Elucidation will potentially provide still more exciting routes for development of small-molecule drugs tailored to evoke a highly specific response.

Long-term therapy with known IR modulators is associated with detrimental side effects that limit their use. Definition of the consequences of ligand binding, in terms of alteration of receptor conformation, may be the key to overcoming this problem. Alterations in receptor conformation will affect both activation of specific transactivation domains within the receptor and interaction of these domains with other proteins required for transcription initiation. The development of screens that detect specific alterations in receptor conformation is critical to the identification of compounds with selective activity.

One approach is the use of assays that identify compounds capable of activating only a subset of receptor transactivation domains. Identification of cells and/or mutant receptors capable of distinguishing compounds that activate via specific transactivation domains can lead to the discovery of agonists or partial agonists with selective activity. The validity of this approach has been demonstrated for ER and may extend to other IRs. Alternatively, as we gain understanding of the role played by accessory factors in the regulation of transcription, assays designed to directly measure productive interaction between these factors and the IRs may aid in the identification of new classes of selective compounds. Recently, accessory proteins have been identified that associate with a number of different IRs.

These include the estrogen receptor associated proteins (ERAPs),[75] additional ER-associated proteins that are involved in the modulation of ER activity, the triiodothyronine receptor auxiliary protein (TRAP),[123] and the 110 kDa receptor accessory factor (RAF)[124] associated with AR. As these and additional, yet to be discovered, factors are characterized and their tissue distributions are determined, their physiological significance can be elucidated. Both functional and biochemical assays dependent on these proteins can then be established and used to discover compounds with selective therapeutic action.

The growing number of IRs with related subtypes provides another means to discover selective small-molecule modulators. To date, four distinct subfamilities of the retinoid receptor family have been identified: RAR, RXR, ROR, and RZR. Each of these subfamilies contains a variable number of subtypes: three RARs, three RXRs, two RZRs, and two RORs. Subfamily-selective compounds, as well as pan agonists, for the RARs and RXRs have been identified. A variety of assays have demonstrated that retinoid subtype-selective compounds lead to different pharmacologies. By exploiting the existence of subtypes in other IR families, compounds may be found that also exhibit differences in pharmacology. The TR and PPAR receptor families both provide exciting targets with potential utility in the treatment of cardiovascular disease and obesity. Since fibrates, which are currently used to reduce triglycerides, modulate the activity of PPARs, more selective compounds may give cleaner pharmacology. PPAR$\gamma$ is activated during the differentiation of adipocytes; modulating its activity may be important in controlling obesity. Thyroid hormone is clearly implicated in thermogenesis, a critical control point in fat deposition and use. In addition, thyroid hormone deficiency leads to elevated serum lipid levels that can be corrected by replacement therapy. The limited use of thyroid hormone in normal individuals due to its associated side effects may be overcome through the development of receptor-selective compounds.

The interaction between ligand-bound receptors and response elements in target genes may provide another means through which selectivity can be achieved. In general, target genes regulated by binding of the RARs and RXRs contain response elements that consist of two directly repeated half-sites. Recent studies have shown that RXR/RAR heterodimers activate transcription in response to *all-trans*-RA or 9-*cis*-RA by binding to direct repeats separated by five base pairs (termed a DR5 element) such that RAR occupies the downstream half-site. RXR homodimers activate transcription in response to 9-*cis*-RA by binding to direct repeats separated by one base pair (a DR1 element). RAR/RXR heterodimers can also bind to DR1 elements, with greater affinity than the RXR homodimer; however, in most contexts RAR/RXR heterodimers are unable to activate transcription in response to either *all-trans*-RA or 9-*cis*-RA. Thus, RARs appear to inhibit RXR-dependent transcription from these sites. RAR can be switched from a retinoid-dependent activator to an inhibitor when it occupies the upstream half-site of the DR1 element. These findings regarding the interaction between the ligand-bound receptors and their response elements clearly demonstrate that receptor conformation and binding characteristics can be manipulated to alter the physiological outcome of receptor binding.

Not only is tissue distribution of the receptor itself a factor in restricted activity but also the availability of heterodimeric partners is critical in determining receptor activity. The role of heterodimer formation between subfamily members of the same IR family in the regulation of target genes has been demonstrated. A body of evidence now exists indicating that heterodimer formation between different intracellular families also plays an important role in the regulation of gene expression. The receptor complement of a particular cell or tissue type will therefore determine not only the response mediated by a given IR family, but may also significantly affect responses mediated by other intracellular superfamily members. As the role of heterodimer formation between superfamily members is examined, the tools developed will allow us to monitor these interactions, which in turn will enable identification of new drug targets.

Lastly, one of the most promising avenues for selective small-molecule drugs is the identification of modulators of the growing number of orphan receptors. The orphan receptors' tissue distributions and patterns of interactions with other IRs indicate that they play important roles in the regulation of gene transcription. Defining their physiological roles is the first step in exploiting these receptors for drug discovery. Identification of ligands, determination of their effects *in vivo*, and production of transgenic mice in which the gene for the orphan receptor is knocked out or overexpressed are ways that this can be accomplished.

The complexity of signal transduction pathways for IR ligands and for extracellular signaling proteins, such as the cytokines, can lead to the discovery of small-molecule modulators via numerous routes. The future challenge resides in the dissection of these pathways and in determining the optimal points of intercession for useful therapeutic outcome. For the IRs, this will require greater understanding of the proteins that transmit information from the IRs to the general transcription apparatus. A greater understanding of the role of IR phosphorylation may also be useful in the identification of new and useful targets for drug discovery. For the JAK/STAT pathways, further insight into the selective modulation of the activity of specific kinases and phosphatases, as well as the myriad protein–protein and protein–DNA interactions involved in signal transduction will be required. The ability to identify small molecules that modulate these activities in the IR and JAK/STAT pathways depends greatly upon the development of high-throughput screens based upon molecular insights into their mechanisms of action. Although this review describes drug discovery approaches based upon modulation of transcriptional activity, additional targets for modulation of steroid hormone action have been identified, particularly the steroid metabolizing enzymes that are known to both activate and inactivate receptor ligands.

## Conclusion

From the perspective of drug discovery, the parallels between IR and STAT signal transduction are clear. Both the STATs and the IRs are latent transcription factors activated by cellular exposure to relevant ligands.

High-throughput, cell-based screens using reporter enzymes can be constructed in which the consequences of transcriptional modulation by potential small molecule agonists of STATs and IRs can be readily assessed. The structures of the reporter plasmids used in the IR and STAT assays are also similar. Each is composed of a reporter enzyme gene under the transcriptional control of a response element in the context of a minimal or naturally responsive promoter. These screens have demonstrated untility in IR drug discovery. The discovery of the JAK/STAT-signaling pathway presents an exciting approach to cytokine-related drug discovery that can yield small-molecule agonists and antagonists with patentability, oral bioavailability, and ease of manufacture. The drug discovery strategies described in this review are designed to identify compounds with novel and therapeutically useful properties.

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