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COMMENTARY

STEROID RECEPTOR STRUCTURE AND ANTIHORMONE DRUG DESIGN

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A number of plant, insect, and vertebrate hormones modulate cell function by binding to protein carriers that are members of a superfamily of receptors capable of acting as transcription-activation signals [1–3]. Whereas the protein hormone receptors are generally localized in the membrane, those for steroids are almost exclusively soluble albeit their exact localization in the nonfunctional state remains debatable [1–3]. When a ligand comes in contact with the hormone binding domain (HBD†), a series of events lead to sequential, selective, and tissue-specific stimulation or suppression of genes that is limited in time and space. Recent identification of disease-specific receptor defects has imbued fresh life into the idea of a molecular solution to hormone based dysfunctions in humans [4, 5].

The quest for agents capable of antagonizing hormone action assumes importance for a number of reasons. First and foremost, antihormones permit molecular dissection of the receptor such that the domain structure within the protein may be correlated with various functions. Second, products with antihormone action may be employed specifically to control hormone-regulated processes with wide-ranging implications in contraception, population control, hypertension, growth and development, and cancer, etc.

This article will review the structure/function basis of receptor-mediated steroid hormone action as a prelude to the development of specific derivatives endowed with antagonist activity. The action of leading antihormone classes will be presented both with biological and clinical implications in an effort to stimulate debate on a future course of action.

Structure–function organization in the steroid receptor family

The primary structure of steroid receptors is organized into several domains with defined functions. The N-terminal region is hypervariable among the 30 or so members that constitute this superfamily of ligand-activated proteins, and largely determines the actual length of the carrier from 984 residues in the mineralocorticoid receptor (MCR) to only 373 in Drosophila embryonic gonad factor. It is immunogenic and contributes to receptor localization as well as trans-activation in a manner that is not clearly understood [2].

The C-terminal HBD consists of approximately 250 residues in the glucocorticoid receptor (GCR) but it may be much shorter in other members of the superfamily [2]. The HBD forms the binding site of a number of cellular constituents such as HSP 90 that control the stability and accessibility of the receptor in the nonfunctional state [6]. Despite the length variation, this region is highly conserved within the 30 or so proteins that include receptors for vertebrate hormones such as steroids, retinoic acid, and thyroxin, as well as a host of insect gene products.

Relatively little information is available regarding the exact binding of the ligand on HBD. In the GCR, the hormone agonist binds to methionine 622 and cystine 754 but in the progesterone receptor (PR) methionine residues 759 and 909 accept the ligand [1–3]. Not much is known regarding the binding of steroids, and of antihormones, to other receptors under these conditions, partly due to the unavailability of suitable probes.

Leucine zippers have been identified in the HBD of all of the receptors and their coiled coil structure appears to be involved in dimer formation [7].

Hormone–receptor interaction induces an allosteric change in conformation, releases the HSP 90 if present, and permits dimerization ($K_d\,10^{-9}$ M) or tetramerization ($K_d\,10^{-11}$ M) that alters complex stability for correct positioning along the DNA [8]. Some of the residues for dimerization have been identified recently in the DNA-binding domain [DBD] of GCR (Fig. 1).

The DBD of about 70 residues is sandwiched between the two termini mentioned above, and is the most conserved of all the domains in the superfamily. Cystine residues in positions 440, 443, 457 and 460 coordinate a zinc atom to form the N-terminal CI finger; the C-terminal CII finger is formed by a similar folding of cystine residues in positions 476, 482, 492 and 495 [9]. Differences of just a few amino acids in the zinc fingers permit the identification of two subfamilies of receptors. Glycine

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† Abbreviations: HBD, hormone binding domain; MCR, mineralocorticoid receptor; GCR, glucocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; ER, estrogen receptor; ERE, estrogen response element; GRE, glucocorticoid response element; HRE, hormone response elements; DBD, DNA-binding domain; TF, transcription factors; TA, triamcinolone acetonide; and AEBS, antiestrogen binding protein.

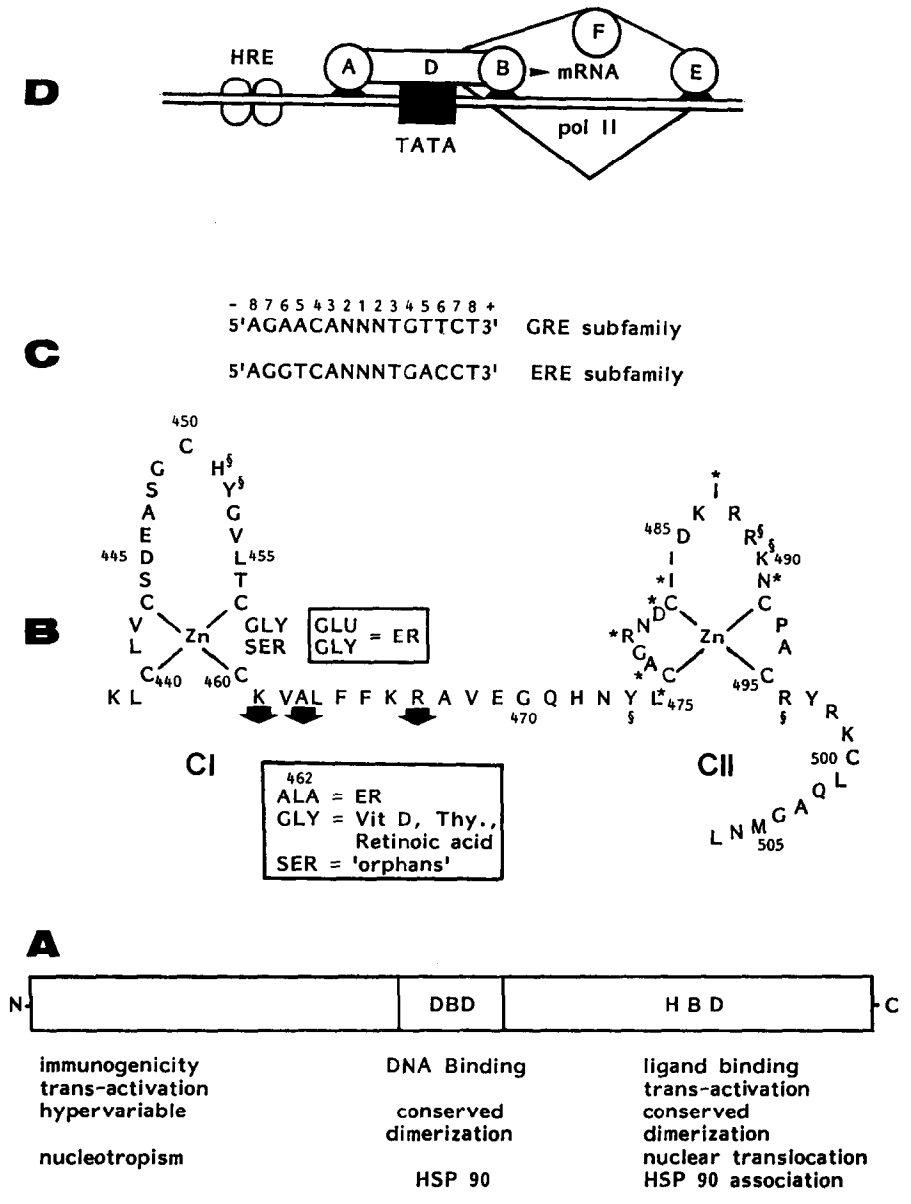

Fig. 1. Schematic representation of steroid receptor-mediated trans-activation. (A) Principal receptor domains exhibit some overlapping functions since specific residues involved in each type of action have not been fully elucidated [1–3]. The zinc finger motive (B) consists of the N-terminal CI finger, formed by residues 440–469, and the C-terminal CII finger, consisting of residues 474–502 (inclusive) in the DBD of rat GCR overexpressed *in vitro* [11] with a total receptor length of 777 amino acids compared to 795 for human GCR [1–3]. The one letter amino acid code has been used throughout except for residues 458, 459 and 462 in the GCR-like P box to distinguish it from the ER-like P box. One single amino acid substitution in 462 leads to either the "orphan" clan, or that for Vitamin D, thyroid hormones and retinoic acid, some ligand-based discrimination is also exerted by residues 476–481 of CII [2]. Dimer interface (*) is assured by residues 475, 477, 479, 481, 483, 487, and 491 for complex stability in the major groove of DNA [11]. The hormone response elements (HRE) are divided into a glucocorticoid response element (GRE) subfamily, consisting of AR, PR, GCR and MCR, and an estrogen response element (ERE) subfamily for other members [2, 10], as shown in (C). Arginine 466 and lysine 461 of CI form hydrogen bonds with G4 and G-7 of GRE, respectively, whereas valine 462 makes a van der Waals' contact with T5 of GRE [11]. Specific (§) phosphate contacts with the DNA are also established via residues 451, 452, 474, 489, 490 and 496 [11]; non-specific contacts have not been indicated. The trans-activation domain in the HRE dimer either stabilizes or catalyzes the assembly of a preinitiation complex at the proximal promotor (TATA box) of the structural gene (D). This involves the transcription factors IIA, IIB, IID, IIE and IIF, permitting the polymerase (pol II) to initiate mRNA synthesis [8, 12]. This schematic representation has been constructed from data currently available only to illustrate the principles of steroid hormone action and does not attempt an exhaustive review.

458, serine 459, and valine 462 characterize the GCR-like subfamily consisting of GCR, MCR, PR, and the androgen receptor (AR). In the much larger estrogen receptor (ER)-like subfamily, these are replaced by glutamic acid, glycine, and alanine, respectively, in the CI zinc finger. Within the ER subfamily, discrimination is assured by a glycine 462 in CI of vitamin D, thyroid, and retinoic acid receptors, or a serine in the "orphan" clan, as well as by changes in residues 476–481 in CII (Fig. 1).

These zinc fingers position the receptor dimer to hormone response elements (HRE) in the target cell DNA [10]. They all carry the consensus 5′-AGGNxCAN(0-3)TGNyCCT-3′ divided into the ER-subfamily if Nx = T and Ny = A, and the GCR-subfamily if Nx = A and Ny = T. Thus, one half site recognition by CI is separated by the mirror image (other half) of the palindrome [9, 10].

Recent crystallographic studies have shown that the CI finger engages in the major groove of DNA containing the HRE, whereas no protein contact is made in the minor groove [11]. More specifically, arginine 466 donates two hydrogen bonds to G(4), valine 462 makes a van der Waals' contact with the methyl group of T(5), and lysine 461 forms two hydrogen bonds with G(−7); a number of phosphate contacts are also established [11]. These studies with the cloned DBD may not exactly duplicate the manner in which the much larger GCR needs to be accommodated in the whole cell *in vivo* since HRE for GCR and PR are identical yet the steroid ligand assures genetic modulation specific to the hormone [2].

The initiation of mRNA synthesis in the mammal is a complex process, involving a number of transcription factors (TF), that is poorly understood (Fig. 1). It has been suggested that TF-IID interacts with the proximal promotor (TATA box) of the structural gene and this complex is subsequently stabilized by the sequential assembly of TF-IIA, TF-IIB, RNA polymerase, and TF-IIE/F [12]. The receptor either stabilizes the preinitiation complex and/or facilitates its assembly via the trans-activating regions both in the N- and C-terminal domains [13].

The remarkable simplicity in receptor organization and gene modulation raises the question of the manner in which the cell is assured of functional diversity. Alternative splicing of large RNA precursors, distinct promotors, redundant genes, and a combination of these, are some of the mechanisms that assure receptor diversity at the level of gene transcription [2].

Post-translational modifications such as phosphorylation have already been well documented [14]. It has been shown recently that consensus sequences identical to carriers that bind ATP are also present in members of the steroid receptor superfamily [15], leading to the demonstration that MCR binds ATP as well as calcium (unpublished observations). The possibility of kinship with other superfamilies and the cross talk between them adds new dimensions to the level of post-translational modifications that assure appropriate response in myriads of physiological situations.

Antihormone action

Historically, it was postulated that antagonists displace the hormone ligand from the active site on the HBD to reverse some or all of the hormone-mediated processes enumerated above. A very large number of molecules was synthesized primarily by the introduction of various groups in the natural or the synthetic agonist with rather unexpected and often curious results.

Mineralocorticoid hormones regulate the hydrosodic balance in the mammal whose dysfunction leads to various pathological manifestations such as hypertension, Conn's syndrome [16]. The hypertensive disease has been related to a receptor defect in the rat [5], and the symptomatic treatment of hypertension by spirolactones via MCR occupancy has attracted much attention [17]. Thus, a very large number of spirolactone derivatives has been synthesized to circumvent gynecomastia, impotence, menstrual abnormalities, etc., associated with administration of canrenone or spironolactone [17]. The mass of available information permits a number of startling conclusions.

No correlation was apparent between the biological potency of various derivatives *in vivo* (Kagawa test), and their affinity for the MCR *in vitro* [18]. Both RU 26752 and ZK 91587 (Fig. 2) rendered the MCR unstable at 37° and interfered with receptor activation [19]. Some organ specific differences were also noted when rat kidney and heart were compared, suggesting post-translational modifications [20]. The experimental hypertension induced by aldosterone could also be reversed by both steroids without any indication of nephrotoxicity [21, 22]. In fact, the affinity and the specificity of these analogs even permitted MCR purification for the very first time [23, 24], and ZK 91587 has actually replaced the natural hormone aldosterone as the "ideal" ligand to study the MCR [16]. However, the experimental potency of RU 26752 in the rat did not match its antimineralocorticoid effect in the human [16]. The antimineralocorticoid research comes full circle here with the availability of only two clinically proven drugs—spironolactone and its metabolite canrenone—despite the fact that they exhibit negligible affinity for the MCR [16, 19].

The interaction of progestins with processes sensitive to aldosterone has a long and often contradictory history. Whereas natural progestogens exert a hypotensive effect *in vivo* [25, 26], synthetic derivatives neither exert an antimineralocorticoid action nor exhibit significant affinity for the MCR [26, 27]. The "ideal" PR ligand R 5020 is nevertheless an important tool for MCR analysis [28], more so because it can be covalently linked to MCR by photo chemistry, thereby providing an important new tool for the analysis of HBD in this receptor [29, 30]. Photosensitive antagonists have hitherto been wanting and may represent an exciting new venue of research for the chemist.

The cross-reactivity between mineralocorticoids and progestins extends even further when glucocorticoids are taken into consideration. Aldosterone exerts GCR-mediated actions [31], hypertension due to glucocorticoids can be reversed by RU 38486 [32],

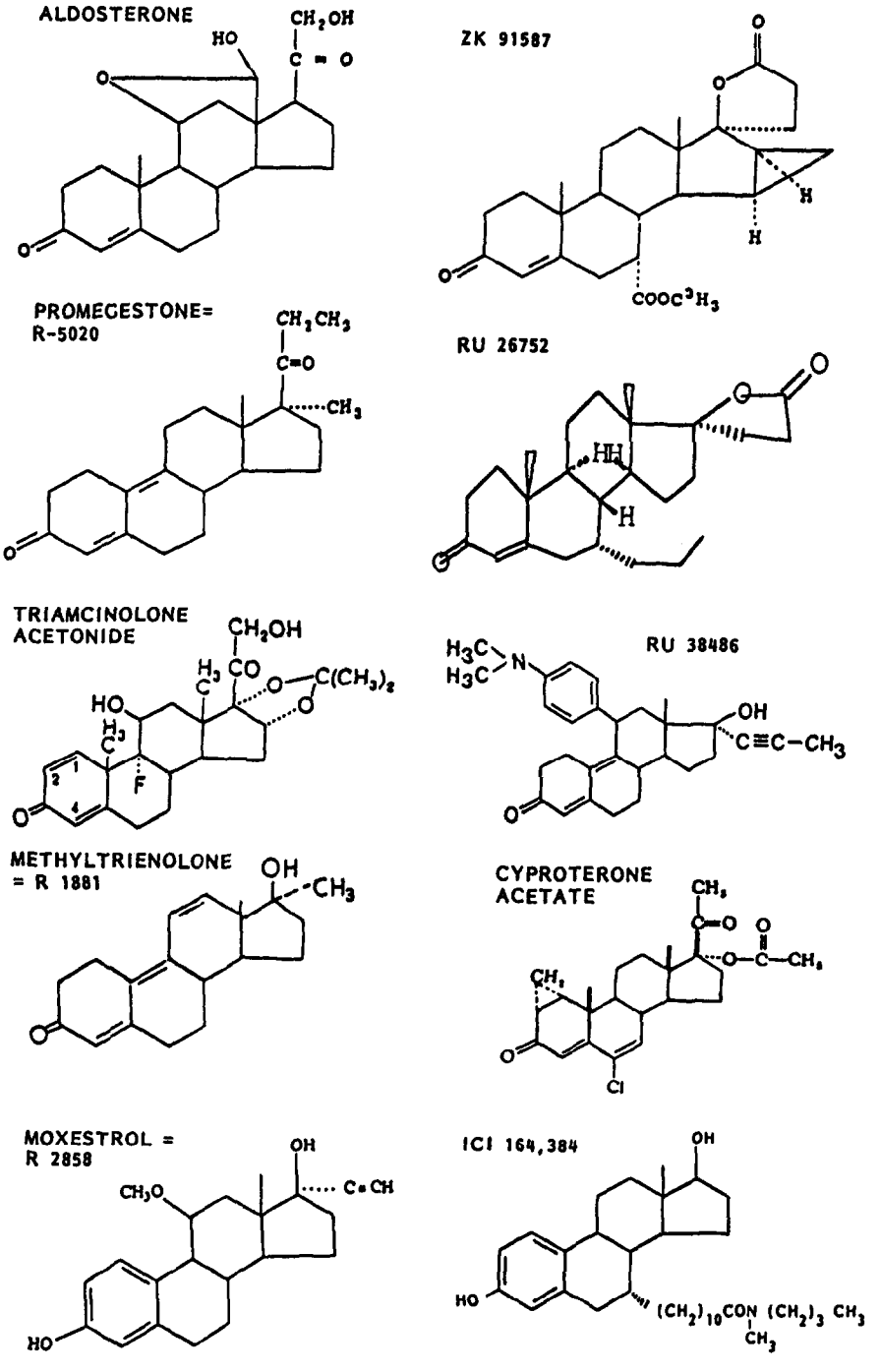

Fig. 2. Principal steroid hormones and leading antagonists. The natural mineralocorticoid aldosterone has been all but replaced by the methoxycarbonyl derivative (ZK 91587) of spirolactone as the ideal, MCR-specific ligand whose comparison with the 7-α propyl derivative (RU 26752) permits MCR cartography [16, 19, 20]. Promegestone is the ligand type for the PR but binds to MCR and can be used effectively for photochemical analysis of both MCR and PR [29, 30]. RU 38486 antagonizes the action of progestins as well as glucocorticoids represented by triamcinolone acetonide [33, 35]. Methyltrienolone (R 1881) forms the ideal ligand for the AR but the widely used androgen antagonist cyproterone acetate is also a potent progestin [46, 47]. ICI 164,384 comes closest to the definition of a specific antiestrogen whose action seems to proceed via the ER that can be labeled best with the synthetic estradiol derivative Moxestrol [50–54].

and some of the progestins exhibit antiglucocorticoid activity [33, 34]. A possible explanation for these intriguing observations was forthcoming by receptor cartography with synthetic derivatives screened for potential antagonist activity [35, 36].

Proline residues in the HBD of GCR and PR are clustered in such a manner as to form a hydrophobic pocket common to these two receptor classes and shared to various degrees by MCR and AR given the conserved primary structure of the C-terminal domain [15]. In fact, the antiglucocorticoid action of RU 38486 is matched by its antiprogestin activity [35, 36]. The former has already been exploited in the clinical treatment of a number of affections such as Cushing's syndrome [37], but the long-term application in glaucoma, immunodeficiency, hypertension, and stress is clearly limited. Unfortunately, the antigestational action of this molecule for the regulation of fertility has aroused much controversy [38], masking its beneficial effects in the control of progestin-dependent syndromes such as neoplasia and sexual aggressivity [35, 36].

Several types of modifications were attempted to synthesize molecules with dissociated and specific antagonist activity. The large C17 propynyl residue in RU 38486 fits into the hydrophobic pocket in GCR and PR. The introduction of a benzyl group in C10 yielded RU 43044 with high affinity for GCR and no affinity for PR. This molecule was six times less effective than RU 38486 as an anti glucocorticoid *in vitro* and exhibited no action *in vivo* due possibly to rapid metabolism which could not be prevented by the introduction of C1 or C6 double bonds [36].

Modifications in the D ring led to the synthesis of RU 46556 and RU 49295 with 17-unsaturated spiroether groups both of which are more potent abortifacients than RU 38486 but their anti-glucocorticoid activity is far less than that of the parent molecule. They also exhibit significant androgenic activity in higher doses, again indicating a conserved domain structure of the receptor [36]. Thus, RU 38486 remains the only viable antagonist that has been commercialized as Mifepristone (anti-GCR action) and Mifegyne (anti-PR activity).

Analysis of the receptor function with the aid of RU 38486 led to some rather unexpected findings. The affinity of Mifepristone for rat liver GCR was less than that of the pure agonist triamcinolone acetonide (TA) but higher than that of dexamethasone for GCR in rat kidney [39], thymus and hepatoma cells [33]. RU 38486 dissociated from the activated GCR much faster than the agonist in thymus and rat liver cytosol *in vitro* [33], contrary to GCR stabilization in the kidney cytosol [39] and in lymphoma cells *in vivo* [40]; PR was also stabilized in the chick oviduct cytosol [41].

Whereas rat liver GCR-RU 38486 complexes could be heat-activated just as well as TA-GCR complexes [42], rat thymocyte GCR–RU 38486 complexes could be activated only partially [35]. Similarly, an idiotype antibody raised against TA-BSA did not recognize RU 38486 bound to rat liver GCR, implying differences in the conformation and topology of the active site [43]. Chick oviduct, too, exhibited two separate binding sites, one each for RU 38486 and progesterone, contrary to the situation in calf uterus cytosol where both ligands saturated an identical receptor [41]. RU 38486–GCR complexes exhibited diminished binding to mammary tumor virus DNA and to thymus nuclei, suggesting that the antagonist impairs some intranuclear events [35].

All these confirm some of the earliest observations regarding receptor multiplicity [44]. The presence of two or more binding sites on the HBD has been suggested by a number of independent studies [16, 19, 20, 45], contrary to the classical notion of a single binding site [1–3]. Furthermore, tissue-specific differences point to subtle post-translational modifications that cannot be delineated merely by studying the cloned product *in vitro*. These considerations should be borne in mind while designing new derivatives with antagonist activity.

Interaction between different receptor classes assumes another dimension of complexity when androgens are considered. The male hormone influences a wide variety of cellular processes in several tissues (kidney, liver, prostate, submaxillary gland) [46]. Thus, potential applications of anti-androgens are vast and include acne, seborrhea, hirsutism, baldness, hypersexuality, and hormone-dependent tumors such as breast and prostate cancer [46].

Methyltrienolone (R 1881) was synthesized as the prototype androgen receptor ligand since the natural hormone testosterone needs to be transformed to the active metabolite 5-α-dihydrotestosterone by a reductase which can be inhibited most effectively by progesterone derivatives [46, 47]. The interaction between AR and PR was even more promiscuous by the fortuitous observation that the progestin cyproterone acetate, synthesized as an oral contraceptive, effectively antagonized a number of responses sensitive to androgens [47]. All of the derivatives screened for antiandrogen action persistently exhibited affinity for PR and no correlation was apparent between antiandrogenicity and binding, activation, or translocation of AR [46, 47]. The androgenicity of progestins even suggested that a functional AR is required for the initiation of PR action [46]. It is recalled here that spirolactones are not only antimineralocorticoids but possess androgenicity as well [16], and antiglucocorticoid action of some of the androgens has also been documented [33].

A screening program for bactericidal and herbicidal agents unexpectedly led to the discovery of the antiandrigenic action of Flutamide and Anandron [48]. These nonsteroidal antiandrogens are devoid of progestomimetic activity, bind AR weakly, and inhibit some of the receptor-mediated events as well as prostate growth [46, 48]. It has been speculated that they may alter phosphorylation in the cell, thereby explaining the anti-AR action [46].

More recently epitestosterone, a metabolite of the natural hormone, was found to exert an antiandrogenic action without progestomimetism, to occupy the AR, and to inhibit the activity of 5-α-reductase, thereby providing a new lead [49].

It is evident from the foregoing that all of the molecules currently being used as antiandrogens suffer from severe side-effects. More important,

they confirm and extend the interrelated nature of receptor-mediated events within the GCR-like subfamily. All evidence suggests that a meaningful search for antihormone action may be targeted at cell specific post-translational events since no correlation is apparent between receptor activity and drug specificity.

The ER-like subfamily of receptors mediates the estrogen action on cell growth, and estrogen antagonists are sought to control hormone-dependent proliferation of tumors in a number of tissues (kidney, prostate, pancreas, gastrointestinal tract, endometrium, mammary glands) [46, 50]. The nonsteroidal agent tamoxifen and its metabolites have literally revolutionized the clinical therapy of receptor positive breast cancer [50, 51]. Their major disadvantage resides in the agonist action exerted under certain conditions, possibly involving the PR [51].

These compounds exhibit weak affinity for the ER and displace the natural hormone from the receptor only at high concentrations; ER translocation, synthesis, and activation are also impaired in a tissue- and species-dependent manner [46, 50, 51]; the ER may be stabilized in a nonfunctional, dimeric form *in vivo* [52], similar to that seen with the PR [41]. Post-translational modifications of ER may be steroid dependent [52], as with MCR [16]. It is unlikely that the antiestrogen binding protein (AEBS) should be a prime target for antiestrogen action and its physiological role remains somewhat of a dilemma [46, 50, 51].

To minimize the side-effects associated with these nonsteroidal antiestrogens, new estrogen analogs have been tested more recently. Of these, ICI 164,384 appears to be most promising with far greater potency than tamoxifen both in binding to ER and in inhibiting cell growth [53], particularly in combination with an LH-RH antagonist [54]. This derivative also suppresses the PR surge seen with other materials [54]. However, the mechanism of action of ICI 164,384 remains to be determined since it permits the activation and subsequent binding of ER to HRE isolated from the vitellogenin gene [54]. It also complexes nonspecifically in rat liver (unpublished observations).

Perspectives and conclusions

Progress in cloning and sequencing the corresponding cDNA has revealed striking similarity in the primary structure of a large number of receptor classes. Prior to the era of genetic engineering, the tedious procedure of classical protein chemistry had revealed some 100 different, enzymatically modified, residues despite the fact that only 20 amino acids are encoded during translation [55–57]. Since covalent modifications of the primary gene product is an obligatory step for the genesis of functionally active proteins, how should the dilemma of such reactions in genetically engineered, recombinant polypeptides be handled? Important differences are known to exist in the processing capabilities of different organisms, as well as different organs and cells in a single organism, depending upon the enzyme distribution pattern for any given class of chemical modification that governs the stability, accessibility, and ligand binding property [55–57].

Perusal of hormone/antihormone action literature clearly reveals organ-, tissue-, and cell-specific differences, most logically due to post-translational modifications. Search for the ideal antihormone would therefore have to extend beyond the ligand binding assays on products obtained by overexpression of cloned genes. As a first step, it would be worthwhile to analyze the extent of secondary modifications by comparing the primary structure of a cloned receptor with that purified by classical methods of protein chemistry. Each secondary modification could be a potential target for antihormone research.

Receptor mutations in defined domains [4, 5] offer another possibility for the analysis of antihormone action, particularly if combined with covalently linked probes that have sadly been wanting. This could be extended even further to include enzymatic mechanisms that alter the availability and activity of steroids *in vivo*. Studies of this type would permit greater definition of receptor structure that could eventually be related to various domain based functions.

Finally, the past decade has seen a literal revolution in the control of hormone-based disorders by receptor-specific ligands. The expected availability of ever more potent products makes it imperative to cope with the ethical, psychological, and legal implications of biomedical progress.

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