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14. ABSTRACT

Our research demonstrated a new protein interaction site on the androgen receptor. We showed that the site called BF3 has the characteristics of functioning in repression and likely binds one or more proteins. We are presently performing experiments to identify these proteins. We used X-ray crystallography to learn the binding mode of small molecule compounds that bind to this site. The BF3 site is allosteric because binding is accompanied by weakening the interaction of the androgen receptor with coactivators. X-ray crystallography demonstrated disorder of coactivator peptides that were well ordered before adding the compounds. Our studies suggest that compounds may be designed to target this site and weaken activity of the androgen receptor. Such compounds could form a new class of chemical therapeutics for treatment of prostate cancer.

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INTRODUCTION

We conducted two types of screens to identify binding of surface compounds to the human androgen receptor (AR). These were complementary. One tested for blocking of coactivator binding and was a functional assay. The second made no assumptions about function but sought compounds that physically would bind and whose structure could be imaged by X-ray crystallography. Together we discovered seven compounds which may be considered prodrug candidates or biological tools. We were surprised to learn that the most potent inhibitors bind to a crevice near helix 8 at a protein interaction site that we termed binding function-3 (BF-3). The database of human mutations showed substitutions of amino acids occurred in humans at BF-3 and that some of these were linked to prostate cancer or androgen insensitivity syndrome. We used X-ray analysis of AR with peptide and DHT bound to show that BF-3 binding compounds weakened the interactions of peptide binding at site AF-2 in the crystalline state.

We believe that BF-3 is an allosteric regulatory site that may be where co chaperones, such as FKBP52 or HSP90 bind *in vivo,* and started a collaboration with Marc Cox at UT El Paso to test these possibilities. Our assays of mutated amino acids forming the BF3 site showed altered function in transcription so that this newly discovered site is a possible pharmaceutical target.

BODY

Original Objectives and Specific Aims
Our objective was to discover molecules that bind AR interaction surfaces) and inhibit assembly of AR protein complexes.

Aim 1: X-ray Screening of Fragments. Soak AR crystals in mixtures of drug-like fragments that are about 200 Daltons and contain sites for later coupling reactions with linkers to combine fragments. Determine three dimensional structures of soaked crystals to locate compounds on the AR surface.

Aim 2: Focused Chemistry. Assemble libraries of computationally designed compounds that will fill the defined sub-pockets in the AF-2 surface. Soak AR crystals in mixes of these compounds and use X-ray crystallography to locate binding compounds, as described for Aim 1.

Aim 3: Identification and Characterizations of Bound Molecules. Positive binding compounds will be identified by direct imaging from Aims 1 and 2, or by deconvolution and additional crystal soaks to determine high resolution structures of compounds attached to AR. Binding interactions and constraints on additions to the bound fragment will be characterized from atomic coordinates.

Aim 4: Linkage Chemistry and Assays. Identified fragments from Aim 1 will be linked by synthetic chemistry to make compounds that will be tested for binding in biochemical and cell assays and by X-ray crystallography. Lead compounds identified in Aim 2 will be tested using the same methodologies.

SUMMARY OF RESULTS

Aims 1 and 2 were completed with compound libraries that included about 4000 compounds derived from computationally filtered libraries and by random selection of compounds of appropriate size and composition that we could purchase or obtain from colleagues.

Aim 3 was completed using X-ray crystallography to image the bound compounds and provide a qualitative degree of binding affinity. The results for Aims 1 through 3 was identification of 7 compounds binding at two surface sites, with 4 compounds binding tightly (Kd ~100 micromolar) and 3 binding weakly (Kd ~ 1-5 mM).

Part of Aim 4, the linkage of positive compounds by chemistry to make molecules that bound more tightly, was abandoned because a synthetic chemistry program could not be completed with appropriate chemistry due to time and financial limitations.

The discovery of BF3 is a major result deriving from this work. We proved that interactions at BF-3 weaken coactivator binding implying that compound or protein binding here promote structural rearrangements in BF-3 that are propagated to AF-2. We clearly proved that BF-3 binding events disorder coregulator peptides that are bound to AF-2. (Estébanez-Perpiñá *et al., PNAS USA*, 2007).

Aim 4. Functional analysis of Binding Function 3:

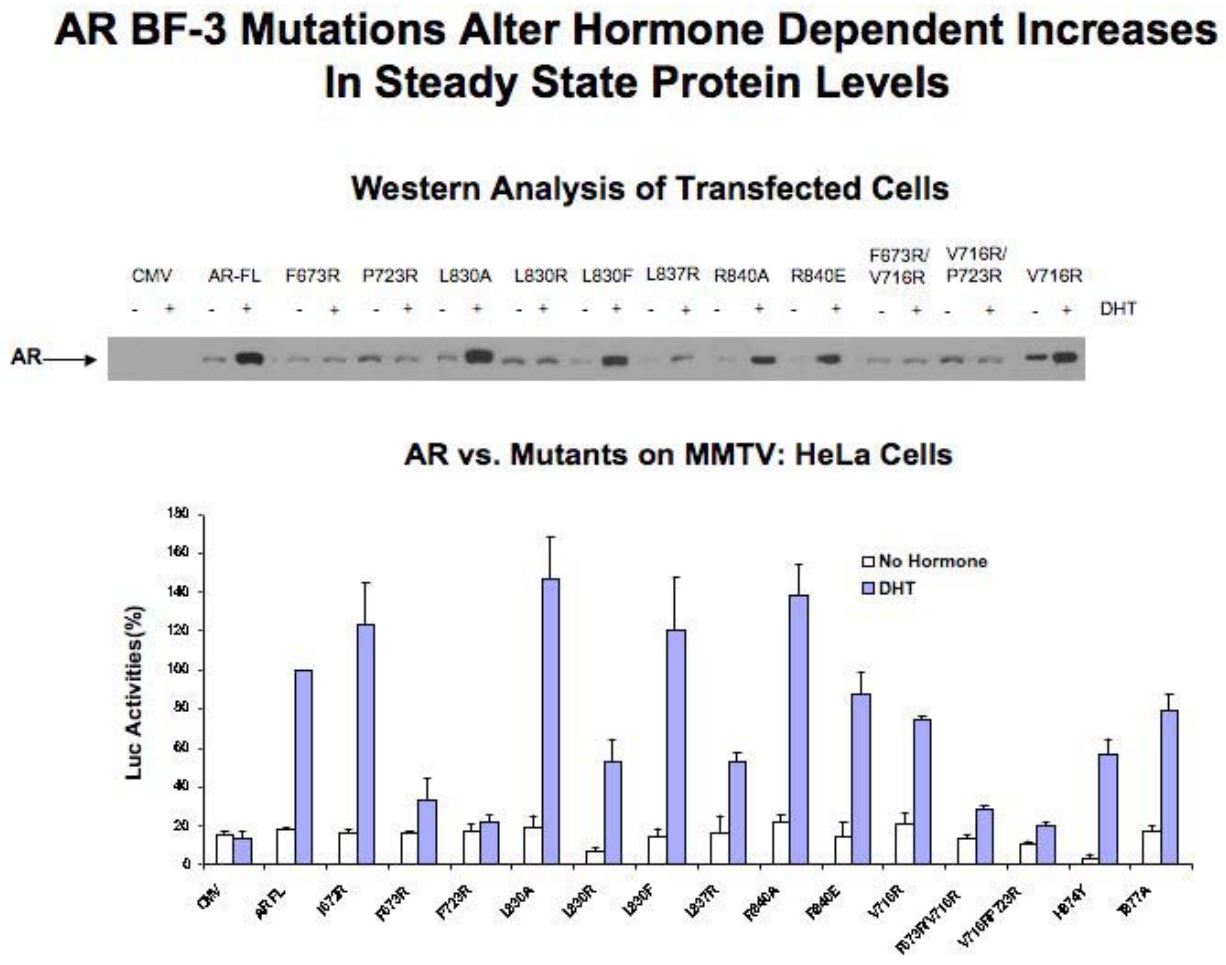
We mutated (Paul Webb, Phuong Nguyen) every amino acid within 5 Å of the positions found for the compounds that we could visualize in BF-3 using crystallography. The mutations were to Ala, conservative, or Arg, disruptive, that inhibit AR activity.

We interrogated the normal role of the AR BF-3 site by targeted mutagenesis and functional analysis of the properties of the mutated receptors. Briefly, we introduced disruptive point mutations into surface exposed residues that contact Triac and FLF in the X-ray structures. We investigated how these mutations affect AR function in cell culture and *in vitro* and whether effects of the mutations are similar to those of drugs that bind the BF-3 surface, Triac and flufenamic acid (FLF).

We assayed activity of 10-20 AR mutants in transfection assays in 10 cell types of prostate and non-prostate origin. Generally, AR BF-3 mutants exhibited diminished androgen response at standard AR regulated reporters, just as Triac and FLF inhibit androgen response by binding to the BF-3 surface. We found an anticipated spectrum of effects. Inhibition was profound, but varied according to the nature of the mutation and the cell type. Certain BF-3 point mutations (e.g. Leu830Arg, Leu837arg) completely inhibit AR activity in PC-3 prostate cells and CV-1 kidney cells. The same mutations inhibited AR activity more modestly (≈50-70%) in HeLa cells. Other BF-3 surface mutations do not have such profound effects. For example, substitution of Leu830 with a small hydrophobic group (ala) enhanced AR activity more than 2-fold relative to wild type control in CV-1 and PC-3 cells. Likewise, substitution of Leu830 with a bulky aromatic group that should fill part of the BF-3 cleft (phe) did not affect AR activity in any cell type. We conclude: i) that BF-3 is essential for normal AR function and that mutations in the AR LBD can profoundly inhibit receptor activity, contrary to existing models which suggest that transcriptional response mostly stems from activation function 1 in the amino terminal domain. ii) that activity of the surface varies according to cell type. iii) that hydrophobic substitutions in the surface often preserve its function.

To determine how BF-3 regulates AR activity we are pursuing a strategy in which we determine effects of diagnostic BF-3 mutations on a variety of known AR properties. For these studies, we rely on matched Leu830 substitution mutations (L830R, L830F, L830A) which display a range of effects on AR activity in transfections (from inactive to superactive) and follow up interesting observations with the entire mutation series. The most striking effect so far is on AR steady state levels. Intracellular levels of AR, as judged by western analysis of transfected cell extracts and stable cells lines that express endogenous ARs, increase in response to hormone treatment. This is a unique feature of AR action, levels of other nuclear hormone receptors such as estrogen receptor and thyroid hormone receptors decrease in the presence of hormone as active receptor is channeled to the proteasome. We find that BF-3 mutations that selectively inhibit AR activity (L830R, L837R etc) also selectively inhibit hormone-dependent accumulation of AR. Again, this effect parallels effects of Triac and FLF, which both inhibit androgen-dependent increases in AR steady state levels.

BF-3 mutations also affect LBD stability *in vitro*. We expressed AR-LBD and versions of AR LBD with BF-3 mutations in *E. coli*; this system is used to prepare AR for biochemical analysis and crystallization. We previously observed that agonist ligand (DHT) must be included in the bacterial growth medium during induction of AR protein expression to prevent irreversible complex formation with bacterial chaperones and concomitant AR protein degradation. We observe that AR LBD bearing an Leu830R mutation (inactive) is difficult to express in the bacterial expression system, even in the presence of DHT, whereas AR LBDs bearing Leu830Ala and Leu830Phe mutations (active) can be expressed. Although we continue to work on this issue, we believe that the results suggest that BF-3 surface mutations may affect the stability and folding of the domain. Our preliminary results are summarized below.



We presently hypothesize that BF-3 is an interaction surface that contacts proteins needed for hormone-dependent stabilization of the LBD. We propose that the BF-3 surface may therefore represent a novel target for drugs that would affect AR activity at a different step to existing drugs, which function by blocking coactivator recruitment.

Plans for the future. We will complete testing the compounds in full length AR constructs and publish the results. We have begun two collaborations, one with Marc Cox on evaluating the BF3 site as a co-chaperone binding site. The second is with Paul Rennie and his colleagues to search for tighter binding compounds and possibly start a medicinal chemistry program to search for better compounds.

KEY RESEARCH ACCOMPLISHMENTS

- Seven compounds were found that bind to surfaces of the human androgen receptor. Cell assays showed that these compounds inhibit transcription in cells in the context of a LBD driving a reporter or of full length AR driving a reporter. Thus, screens developed by this grant led to new compounds that may be chemotypes for new classes of AR inhibitors.

- Discovery of an allosteric effector site of the androgen receptor that has characteristics of a repressor function. Binding at BF3 alters structure disordering coregulator domains bound at AF-2.

REPORTABLE OUTCOMES

Publications

1. Estébanez-Perpiñá, E., Arnold, L.A., Mar, E., Bateman, R., Shokat, K., Guy, R. K. and Fletterick, R.J. (2007) A surface on the androgen receptor that allosterically regulates coactivator binding. *Proc Natl Acad Sci U S A* (*PNAS*), Oct 9; 104(41):16074-9.

2. Estébanez-Perpiñá, E., Arnold, L.A., Jouravel, N., Togashi, M., Blethrow J., Mar, E., Nguyen P., Phillips K.J., Baxter, J.D., Webb, P., Guy, R. K. and Fletterick, R.J. (2007) Structural Insight into the Mode of action of a direct inhibitor of coregulator binding to the thyroid hormone receptor. *Molecular Endocrinology*, Volume: 21 Issue: 12 Pages: 2919-2928 DEC 2007

3. Estébanez-Perpiñá, E., Jouravel, N., and Fletterick, R.J. (2007) Perspectives on designs of anti-androgens for prostate cancer. *Expert Opinion in Drug Discovery*, Volume 2, Number 10, pp. 1341-1355(15) October 2007.

4. Estébanez-Perpiñá, E., Arnold, L.A., Baxter, J.D., Webb, P., Guy, R. K. and Fletterick, R.J. (2008) Developing therapeutic agents for androgen-independent prostate cancer. Invited review, *Nuclear Receptor Signaling* (*NURSA*), In Press.

5. Estébanez-Perpiñá, E., and R.J. Fletterick. The Androgen Receptor Coactivator Binding Interface. Invited book chapter by Springer Science. In Press, 2008.

Personnel receiving pay from the research effort:

Robert J. Fletterick (PI), Peter Hwang (Associate Research Biochemist)

(Note: we are in a no-cost extension period from 9.1.08 – 8.31.09):

CONCLUSIONS

We identified organic molecules that bind to the AR LBD surface. We showed that binding compounds at the surface affects AR function in crystals and in cells.

The discovery of site BF3 has been noted by many scientists in the field of nuclear receptors and has inspired androgen receptor researchers to search for compounds that bind to BF3 and affect activity of the receptor. One study is advanced at Shifa Biomedical Sciences, Philadelphia, PA (Sherin Abdel Meguid) and another is in progress at the Prostate Center Vancouver General Hospital (Dr. Paul Rennie). Perhaps research from these and our group will permit identification of binding molecules that become lead compounds in drug discovery.

Plans for further work: We are working with Prof. Marc Cox and Dr. Paul Rennie to identify proteins that bind to BF3 so that we may form crystals of the receptor with these proteins and learn more about function of the human androgen receptor.