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TITLE: Multivalent Peptidomimetic Conjugates as Inhibitors of Androgen Receptor Function in Therapy Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Kent Kirshenbaum

CONTRACTING ORGANIZATION: New York University
NEW YORK, NY 10012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Androgens are hormones that play a critical role in stimulating prostate cancer growth. Androgens activate a protein called the androgen receptor (AR), which regulates genes involved in cell growth. Although powerful anti-androgen drugs can be administered to block AR action and have been used successfully to treat patients with prostate cancer, over time the tumors become resistant to the drugs, leaving few treatment options. The goal of this proposal is to develop a new approach to block AR activity and stop prostate cancer growth using a new family of molecules called multivalent peptidomimetic conjugates. To accomplish our goals, we will create a set of conjugates with anti-androgens linked to the peptidomimetic backbone at variable intervals along the molecular chain. We will test these molecules for their ability to bind to AR. Those that bind tightly will then be tested in tumor models to evaluate if they block androgen-dependent prostate cancer cell growth. To understand how these molecules block AR function, we will determine the three-dimensional structure of AR bound to the peptidomimetic conjugates. These studies will be used to guide our ability to tailor the conjugates for optimal interactions with the AR.

15. SUBJECT TERMS
androgen receptor, prostate cancer, peptidomimetic conjugates

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TABLE OF CONTENTS:

1. INTRODUCTION:

Androgens are hormones that play a critical role in stimulating prostate cancer growth. Androgens activate a protein called the androgen receptor (AR), which regulates genes involved in cell growth. Although powerful anti-androgen drugs can be administered to block AR action and have been used successfully to treat patients with prostate cancer, over time the tumors become resistant to the drugs, leaving few treatment options. The goal of this proposal was to develop a new approach to block AR activity and to stop prostate cancer growth using a new family of molecules called Multivalent Peptidomimetic Conjugates (MPCs). To accomplish our goals, we created a set of conjugates with anti-androgens linked to the peptidomimetic backbone at variable specified positions along the molecular chain. We tested these molecules for their ability to bind to AR. Those that bind tightly were tested in tumor models to evaluate how they block androgen-dependent prostate cancer cell growth. To understand how these molecules block AR function, we sought to determine the three-dimensional structure of AR bound to the peptidomimetic conjugates. Our results will be used to guide our ability to tailor the conjugates for optimal interactions with the AR.

2. KEYWORDS:

KEYWORDS: Androgen receptor, prostate cancer, peptidomimetic conjugates

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The major goals of the project were:

a) Synthesize a family of multivalent peptidomimetic conjugates
b) Test peptidomimetic conjugates in a series of in vitro and cell-based assays
c) Conduct studies of pharmacological potential through *in vivo* mouse xenograft and PK/PD studies
d) Establish the mechanism of action of peptidomimetic conjugates on the Androgen Receptor through biophysical and X-ray crystallographic studies.

What was accomplished under these goals?

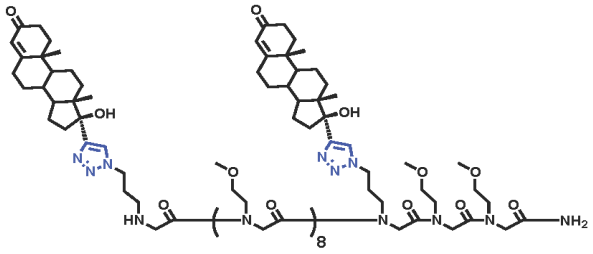
All of the major goals of the project (a-d) were accomplished during the period of the award, as detailed below. Goal (d) was partially accomplished, however, as we were unable to obtain co-crystals of the Androgen Receptor and the peptidomimetic conjugates. Nevertheless, we did expand our understanding of the mechanism of action of the conjugates. Overall, through the successful completion of our major goals, we were able to make substantial progress towards the development of the multivalent conjugates as therapeutics for treatment of late stage prostate cancer.

- **Major Task 1:** Synthesize a family of multivalent peptidomimetic conjugates.
  Responsible PI: Kirshenbaum/NYU Chemistry
  This major task was completed as proposed.

- Subtask 1: Design, synthesize, purify and characterize a family of peptidomimetic oligomer conjugates

  Our initial efforts involved synthesizing a multivalent conjugate presenting two ethisterone ligands arrayed as spatially defined pendant groups on a peptoid oligomer. The conjugate, named Multivalent Peptoid Conjugate 6 (MPC6), potently suppressed the proliferation of multiple AR-expressing prostate cancer cell lines including those that failed to respond to enzalutamide and ARN509 (Scheme 1 & Figure 1).

Scheme 1. The divalent ethisterone conjugate MPC6, a potent modulator of Androgen Receptor activity.



Details regarding the synthesis and the antiproliferative activity of MPC6 were published in our 2016 *Cancer Research* paper (attached).

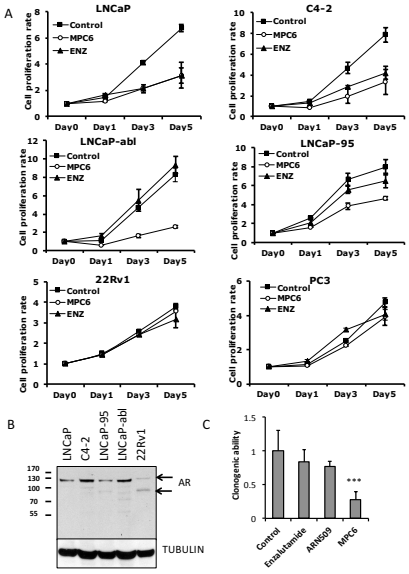


Figure 1. MPC6 inhibit proliferation of multiple AR-expressing prostate cancer cell lines.
A) AR-expressing prostate cancer cell lines (LNCaP, LNCaP-C4-2, LNCaP-abl, LNCaP-95 and 22Rv1) and AR-negative PC3 cells were treated with control or a single dose of 10 μM MPC6 for 5 days and cell growth measured. B) Western blot of AR from prostate cancer cells C) Colony formation in LNCaP-abl cells, treated with vehicle, 10 μM enzalutamide, 10 μM ARN509, or 10 μM MPC6, were measured by a clonogenic assay. ***p=0.0003.

Using the promising activity of a MPC6 as a starting point, we designed and synthesized a series of analogs. This was conducted to meet two objectives: to understand the critical structural features necessary to provide potent anti-proliferative activity; and to identify more active compounds that will facilitate eventual clinical implementation that will advance prostate cancer therapy.

A variety of different analogs were synthesized, yielding 25 novel chemical structures. All of these structures feature multivalent displays of ligands to the Androgen Receptor presented on a peptoid oligomer scaffold. Representative chemical structures are included as a pdf file supplement to this report (see Figures S1 to S9).

The structures include:

- Variations of ethisterone conjugate MPC6 bearing peptide appendages to enable secondary site binding to the Androgen Receptor
- Variations of ethisterone conjugate MPC6 incorporating longer linker groups between steroidal groups and the peptoid backbone.
- Variations of ethisterone conjugate MPC6 incorporating trivalent and tetravalent displays of ethisterone
- Variations of trivalent and tetravalent ethisterone displays incorporating an N-terminal acetyl group.
- Variations of trivalent and tetravalent ethisterone displays incorporating an extended linker group.
- Variations of trivalent and tetravalent ethisterone displays incorporating an N-terminal acetyl group and an extended linker group.
- Variation of divalent ethisterone display MPC6 incorporating N-terminal PEG groups of variable lengths.
- Variation of trivalent ethisterone display incorporating PEG-like linkers between steroidal group and peptoid oligomer.
- Divalent displays of alternative AR antagonist ligands.
- Divalent displays of alternative AR antagonist ligands incorporating PEG-like linkers to peptoid scaffold

We have now established a capacity to synthesize Mutivalent Peptoid Conjugates featuring an array of different chemical modifications. Many of these multivalent peptoid conjugates have been subjected to testing of biological activity, as described in Major Task 2, below. We have been able to identify structural variations that we can associate with enhanced activity.

Major Task 2: Test the peptidomimetic conjugates in a series of in vitro and cell-based assays.

Responsible PI: Garabedian/NYU Med.
This major task was completed as proposed.

Subtask 2: Conduct *in vitro* activity assays
This subtask was completed as proposed.

- We determined the ability of the peptidomimetic conjugates to compete for DHT-binding to the AR LBD in vitro.

- We determined the peptidomimetic conjugates' impact on coactivator peptide binding in vitro.

Subtask 3: Conduct cell-based activity assays
This subtask was completed as proposed.

- We examined the ability of the peptidomimetic conjugates to modulate AR-dependent transcriptional activity.

- We determined the ability of the peptidomimetic conjugates to promote AR-YFP nuclear localization and to block DHT-dependent AR nuclear localization.

- We validated the impact of peptidomimetic conjugates on AR transcriptional activation of endogenous AR target genes by qPCR, and the recruitment of AR to targets by ChIP.

- We evaluated the ability of the peptidomimetic conjugates to inhibit the proliferation of therapy-resistant prostate cancer cells.

We also evaluated the new synthesized MPC6 derivatives synthesized in Major Task 1 that include diverse linkers and peptoid backbone topology by testing them in cell based proliferation assays (Figure 2A-C). We found that increased spacing between ethisterone moieties and changes in peptoid topology eliminated the anti-proliferative effect of MCP6 in therapy resistant prostate cancer, suggesting that both ethisterone ligand presentation and scaffold characteristics contribute to MPC6 activity. We also showed that compared to MPC6, the ethisterone ligand alone failed inhibit cell proliferation at the maximal concentration tested, suggesting that multivalency plays an important role in the activity of MPC6 (Figure 2D).

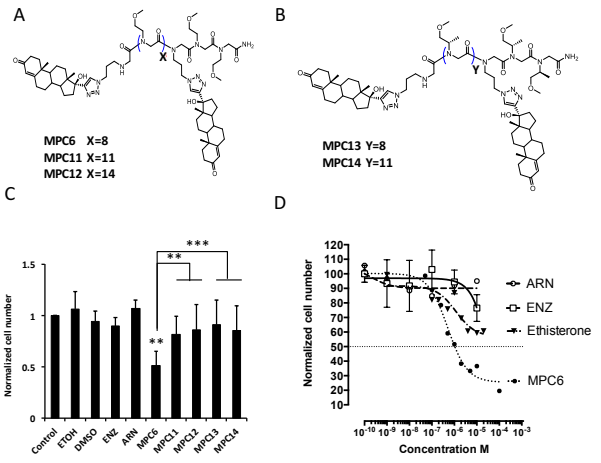


Figure 2. MPCs impact the proliferation of LNCaP-abl cells.
A) Chemical structures of linear divalent ethisterone-peptoid conjugates MPC6, MPC11 and MPC12, spaced by 8, 11 and 14 monomers, respectively. B) Chemical structures of divalent ethisterone-peptoid conjugates MPC14 and MPC15 with a methyl group on peptoid backbone. C) LNCaP-abl cells were treated with 10 µM of vehicle (Ethanol; EtOH, or DMSO), enzalutamide (ENZ), ARN509, or MPCs for 72 hours and cell proliferation measured. The error bar represents standard deviation from six independent experiments. **p<0.01, ***p<0.001. D) LNCaP-abl cells were treated with the indicated compounds and concentrations. After 72 hours, cell proliferation was measured and the EC_{50} calculated. Each point represents a mean value of three independent experiments. Error bars = standard deviation.

We determined that MPC6 promoted AR nuclear localization (Figure 3A). We also found that MPC6 was able to compete for co-activator peptide binding in vitro using a time resolved fluorescence resonance energy transfer (TR-FRET) assay (Figure 3B).

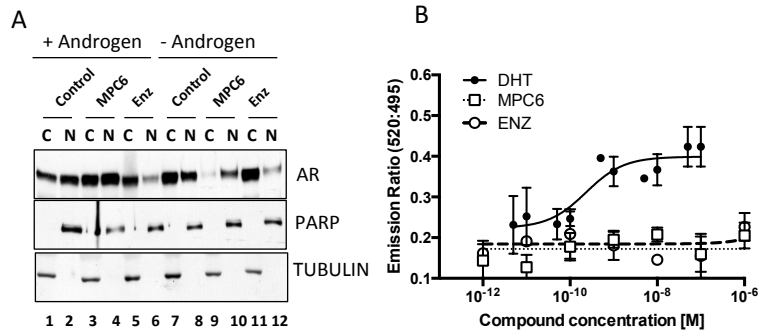


Figure 3. MPC6 promotes AR nuclear localization and blocked AR-coactivator binding
A) LNCaP-abl cells cultured in the presence or absence of androgen were treated with vehicle, 10 µM MPC6, or 10 µM enzalutamide overnight. Western blot shows AR expression in cytoplasm (C) and nucleus (N). B) *In vitro* TR-FRET analysis of the interaction between GST-tagged AR-LBD, terbium-labeled anti-GST antibody, and fluorescein-labeled AR FxxLF co-activator peptide. Titration of DHT, enzalutamide (ENZ), and MPC6 for 10 nM DHT bound AR.

Our studies revealed that MPC6 reduced the expression of the key AR target gene UBCEC2 involved in prostate cancer cell proliferation (Figure 4). These results were reported in *Cancer Research*.

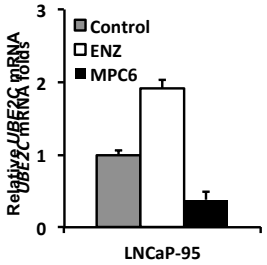


Figure 4. MPC6 decreased AR-V7 expression and AR-V7 target gene transcription in prostate cancer cells.
LNCaP-95 cells were treated with vehicle, 10 µM MPC6, or 10 µM enzalutamide for 48 hours. Total RNA was extracted, reverse transcribed and qRT-PCR performed to assess *UBE2C* mRNA expression. **p<0.01.

Major Task 3: Conduct studies of pharmacological potential through *in vivo* mouse xenograft and PK/PD studies

Responsible PI: Garabedian/NYU Med.

This major task was completed as proposed.

Subtask 1: Demonstrate pharmacological potential through *in vivo* mouse xenograft studies.

This subtask was completed as proposed.

We have been able to confirm and extend our initial finding that MPC6, administered to mice *i.p.*, can significantly inhibit the growth of human prostate cancer tumor xenografts. We have now demonstrated diminished tumor growth in LNCaP-95 xenografts administered at 50 mg/kg (Figure x).

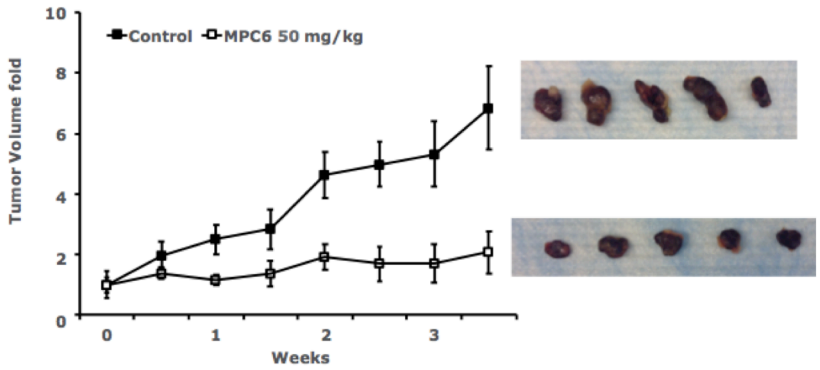


Figure 5:
MPC6 diminishes tumor growth in LNCaP-95 xenografts. Compound was administered IP at 50 mg/kg 4 times/week for 3 weeks.

Subtask 2: Conduct preliminary evaluation of pharmacological properties.

This subtask was completed as proposed.

We established that MPC6 has favorable pharmacological properties:

- MPC6 possess excellent microsomal stability (measure of hepatic clearance) $CL_{int(mic)} < 9.6$ µl/min/mg and a $T_{1/2}$ of >145 min
- MPC6 is stable in plasma and does not exhibit significant chemical alteration after 2 hr at 37 C.
- There is no significant inhibition of critical CYP450 enzymes with an $IC_{50} >$ 50 mM for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4-M
- Good solubility in PBS
- However, our studies did indicate poor oral bioavailability, suggesting that administration to patients may be restricted to IV.

Major Task 4: Establish the mechanism of action of peptidomimetic conjugates on the Androgen Receptor through biophysical and X-ray crystallographic studies.

Responsible PIs: Shared Garabedian/Kirshenbaum/Nettles NYU/NYU Med/TSRI

This major task was conducted as proposed.

We were able to elucidate critical aspects regarding the mechanism of action of MPC6 and its interaction with the Androgen Receptor. We developed an understanding of the role of MPC6 in altering AR cellular localization and in the blockade of co-activator protein binding. We also attempted to conduct X-ray crystallographic studies to observe the binding mode of MPC6 to AR. Although we made several attempts to co-crystallize MPC6 with AR, these proved to be unsuccessful. We then purified AR bound to DHT and attempted to exchange the ligand with MPC6, but the resulting crystal structure contained DHT.

What opportunities for training and professional development did the project provide?

Two post-doctoral scientists in the Kirshenbaum lab received training in bioconjugate chemistry and molecular pharmacology. This training is highly complementary to their intended interest to pursue professional opportunities as independent researchers. One of these post-doctoral scholars, Dr. Dehigaspitiya, is currently applying for faculty positions.

A post-doctoral doctoral in the Garabedian lab, Dr. Yu Wang, received training in cellular and mouse models of prostate cancer. He also was given the opportunity through separate institutional funds to further his education by obtaining a masters in bioinformatics from an on-line program at the University of Indiana. This allowed Dr. Wang to leverage his training to secure a job at a foundation curating patient data for a rare cancer, and interfacing with pharmaceutical companies and the FDA to establish clinical trials for this disease..

How were the results disseminated to communities of interest?

We published a paper on this topic in the journal *Cancer Research*, which is widely read by basic and clinical oncologists. The study was also highlighted in the journal *Nature Reviews Urology*, which targets Urologists in clinical practice. We are assembling data from our recently completed studies in order to publish an article reporting our additional results. We have reported extensively on our results at scientific meetings, addressing both the community of cancer researchers and the community of researchers in molecular pharmacology.

4. IMPACT:

Our work describes the biological evaluation of a new set of multivalent peptoid conjugates (MPCs) We are discovering the therapeutic potential of an innovative new family of compounds against late-stage prostate cancer that is resistant to current treatments.

What was the impact on technology transfer?

We are continuing to work with NYU's Office of Therapeutics Alliances to develop a patent portfolio covering the technologies relevant to multivalent conjugates targeting the Androgen Receptor. We have filed a patent application related to this technology, "Multivalent Peptoid Oligomers, Pharmaceutical Compositions and Methods of Using Same".

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

Wang Y, Dehigaspitiya DC, Levine PM, Profit AA, Haugbro M, Imberg-Kazdan K, Logan SK, Kirshenbaum K, Garabedian MJ. Multivalent Peptoid Conjugates Which Overcome Enzalutamide Resistance in Prostate Cancer Cells. Cancer Res. 2016 Aug 3. PMID:2748852

Thomas, C., Fitting to overcome enzalutamide resistance, *Nature Reviews Urology* (2016) doi:10.1038/nrurol.2016.160 Published online 23 August 2016

7: PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

PIs: Name: Kent Kirshenbaum, PhD
Project Role: Initiating PI.
Nearest person month worked: 3.5
Contribution to Project:
Dr. Kirshenbaum conceived of the chemical platform and helped with the design and synthesis of the peptoid conjugates. He analyzed the experiments involving the chemical synthesis, and the cellular studies of MPC6 and its derivatives. He assisted in authoring the recent paper on MPC6 function and activity in prostate cancer
Funding Support: CDMRP

Name: Michael Garabedian, PhD.
Project Role: Partnering PI.
Nearest person month worked: 3
Contribution to Project:
Dr. Garabedian helped design and analyze the experiments involving the cell based proliferation assays and in vitro biochemical studies on MPC6 on AR. He also helped write the paper on MPC6 function and activity in prostate cancer.
Funding Support: CDMRP

Name: Kendall Nettles, PhD.
Project Role: Partnering PI.
Nearest person month worked: 3
Contribution to Project:
Dr. Nettles is performing the biophysical studies on the AR and MPC6. He is also involved in the DMPK studies.
Funding Support: CDMRP

Post docs and students

Name: Yu Wang, PhD
Project Role: Post doc
Nearest person month worked: 12
Contribution to Project:
Dr. Wang performed the experiments involving the cell based proliferation assays and in vitro biochemical studies on MPC6.
Funding Support: Prostate Cancer Foundation Young Investigator Awardee

Name: Amanda Kapser, PhD
Project Role: Post doc
Nearest person months worked: 20
Contribution to Project:
Dr. Kapser performed the synthesis of MPC6 and its derivatives.
Funding Support: CDMRP, NSF

Name: Harrison Bergman
Project Role: Technician
Nearest person months worked: 10
Contribution to Project:
Mr. Harrison conducted chemical synthesis and purification
Funding Support: CDMRP

Name: Dilani C. Dehigaspitiya, PhD
Project Role: Post doc
Nearest person months worked: 3
Contribution to Project:
Dr. Dehigaspitiya performed the synthesis of MPC6.
Funding Support: NSF

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8 SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: For this collaborative award, independent reports are provided from BOTH the Initiating PI and the Collaborating/Partnering PIs. Tasks are clearly marked with the responsible PI and research site. A report is being submitted to https://ers.amedd.army.mil for each unique award.

9 APPENDICES: see attached documents

Wang Y, Dehigaspitiya DC, Levine PM, Profit AA, Haugbro M, Imberg-Kazdan K, Logan SK, Kirshenbaum K, Garabedian MJ. Multivalent Peptoid Conjugates Which Overcome Enzalutamide Resistance in Prostate Cancer Cells. Cancer Res. 2016 Aug 3. PMID:2748852

Thomas, C., Fitting to overcome enzalutamide resistance, *Nature Reviews Urology* (2016) doi:10.1038/nrurol.2016.160 Published online 23 August 2016

Report supplement – List of chemical structures of Multivalent Peptoid Conjugates (see following pages).

Appendix - Supplement to Progress Report

Kent Kirshenbaum, New York University, Dept. of Chemistry

Multivalent Peptidomimetic Conjugates as Inhibitors of Androgen Receptor Function in Therapy Resistant Prostate Cancer
Garabedian, Kirshenbaum, Nettles
Contract # W81XWH-15-1-0590

List of chemical structures synthesized in support of project.
Responsible PI: Kirshenbaum/NYU

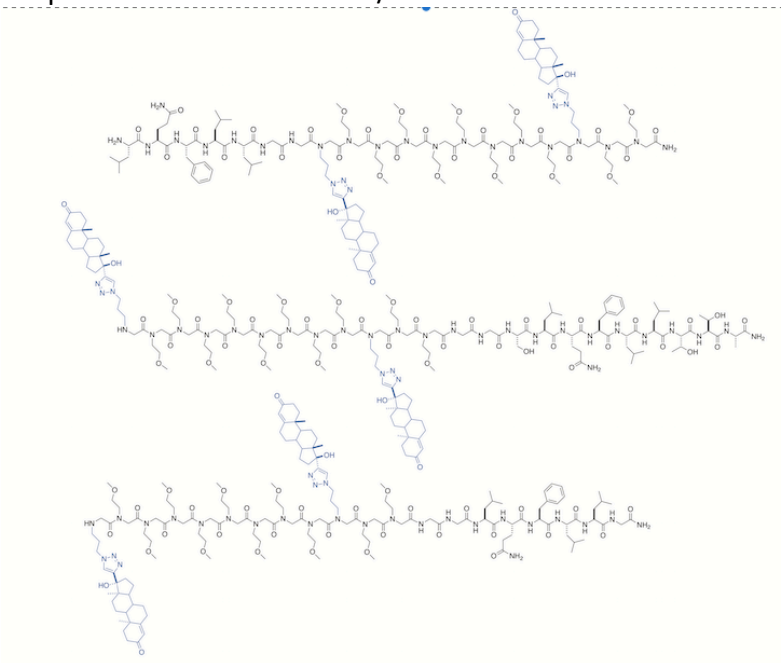


Figure S1:
Variations of ethisterone conjugate MPC6 bearing peptide appendages to enable secondary site binding to the Androgen Receptor

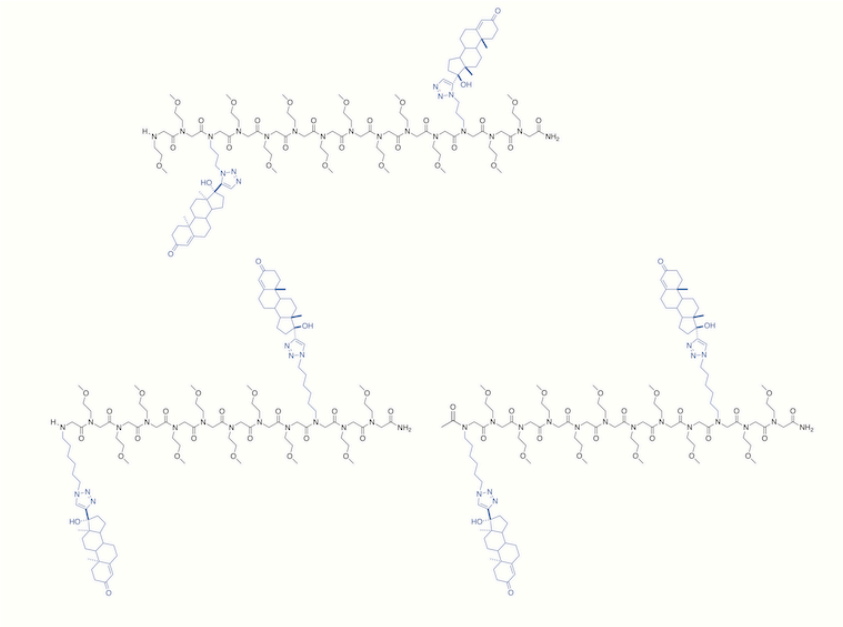


Figure S2:
Variations of ethisterone conjugate MPC6 incorporating longer linker groups between steroidal groups and peptoid backbone.

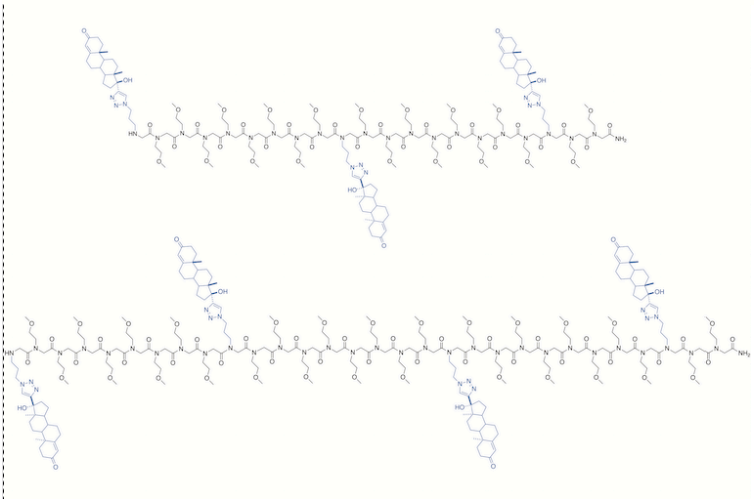


Figure S3:
Variations of ethisterone conjugate MPC6 incorporating trivalent and tetravalent displays of ethisterone.

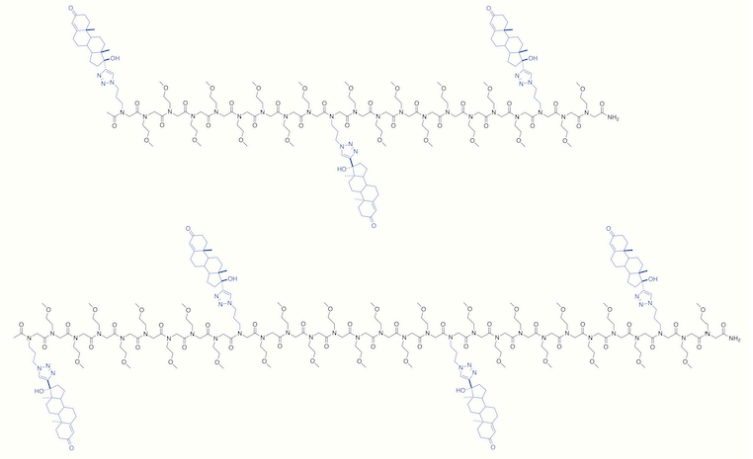


Figure S4:
Variations of trivalent and tetravalent ethisterone displays incorporating an N-terminal acetyl group.

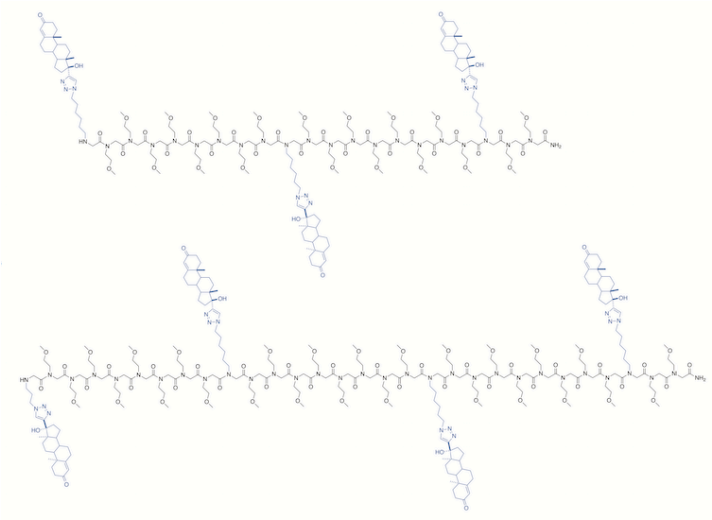


Figure S5:
Variations of trivalent and tetravalent ethisterone displays incorporating an extended linker group.

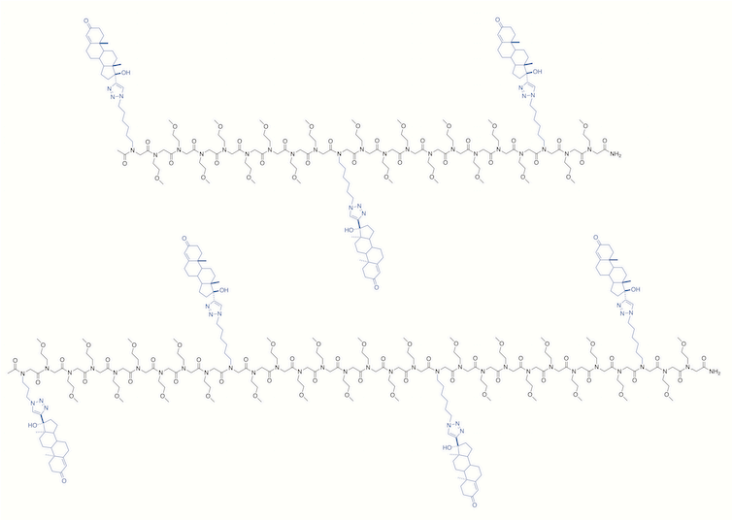


Figure S6:
Variations of trivalent and tetravalent ethisterone displays incorporating an N-terminal acetyl group and an extended linker group.

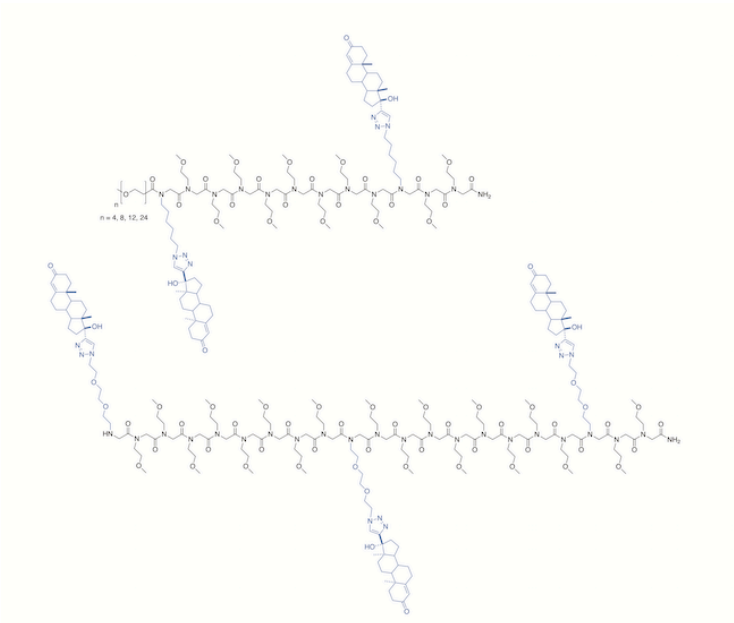


Figure S7:
Top: Variation of divalent ethisterone display MPC6 incorporating N-terminal PEG groups of variable length (4 molecules).
Bottom: Variation of trivalent ethisterone display incorporating PEG-like linkers between steroidal group and peptoid oligomer .

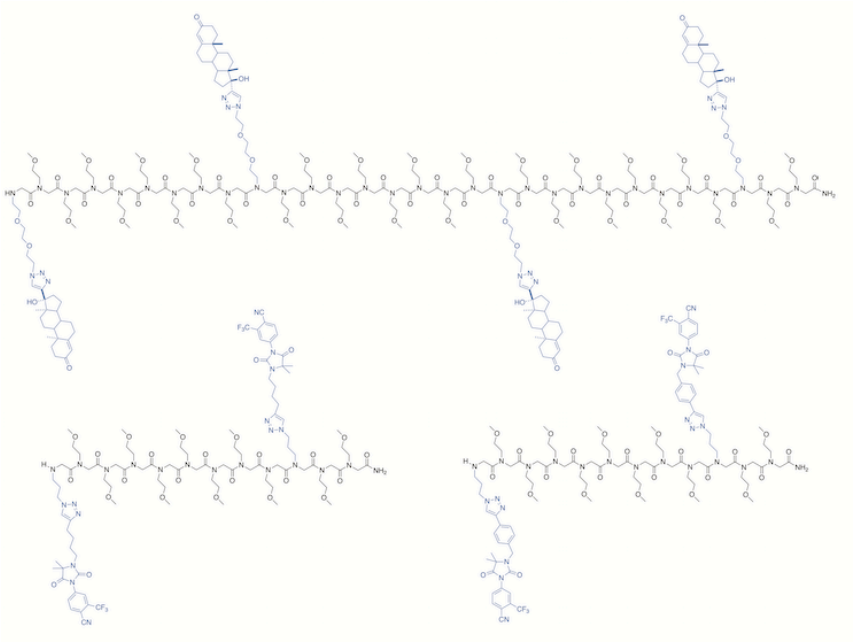


Figure S8:
Top: Variation of tetravalent ethisterone display Bottom: Variation of trivalent ethisterone display incorporating PEG-like linkers between steroidal group and peptoid oligomer
Bottom: Divalent displays of alternative AR antagonist ligands.

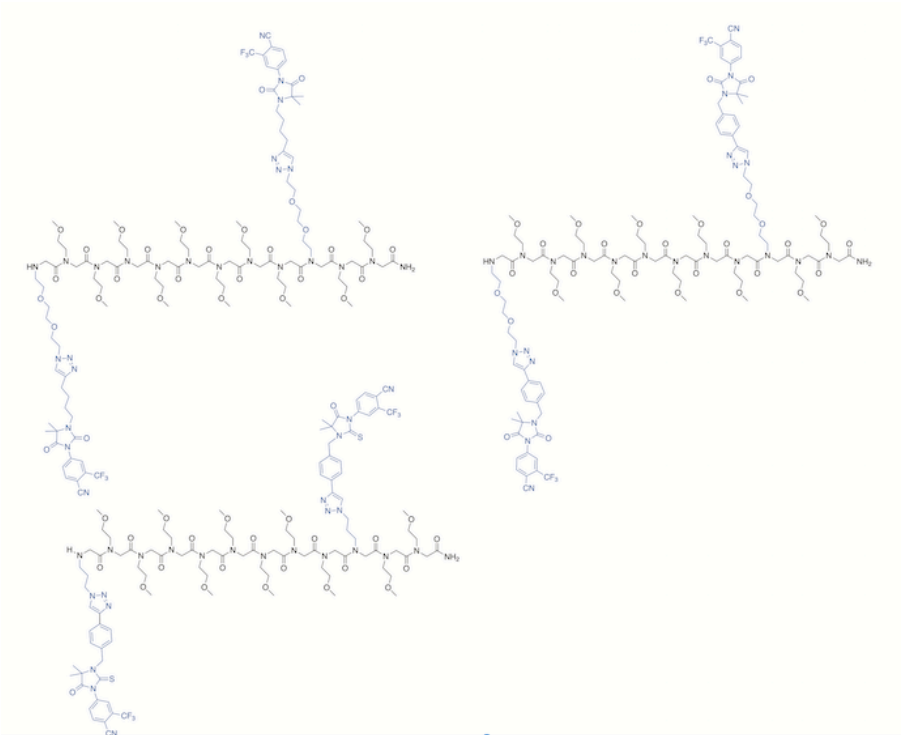


Figure S9:
Divalent displays of alternative AR antagonist ligands incorporating PEG-like linkers to peptoid scaffold.