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PRINCIPAL INVESTIGATOR: Donald W. Kufe, M.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

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14. ABSTRACT

The MUC1 oncoprotein is overexpressed in prostate cancers with aggressive clinicopathologic features and is associated with an elevated risk of recurrence. Our hypothesis is that MUC1 is of functional importance to the development of aggressive prostate cancer and that this effect is mediated through interaction with the androgen receptor (AR). The Specific Aims of this Idea Award are: 1) To define the interaction between MUC1 and AR in vitro and in prostate cancer cells; 2) To assess the effects of MUC1 on AR-mediated gene expression; and 3) To define the effects of MUC1 on growth and survival of prostate cancer cells in response to androgen stimulation and treatment with antiandrogens. Work supported by the first year of this Award has demonstrated that the MUC1-C receptor constitutively associates with AR in androgen-unresponsive prostate cancer cells. The MUC1-C cytoplasmic domain (MUC1-CD) binds directly to the AR DNA binding domain. Specific sequences in MUC1-CD that confer the interaction have been identified and used to identify drugs that block binding to AR. One lead agent has been found to induce death of androgen-unresponsive, and not –responsive, prostate cancer cells. These findings, based on the results of MUC1-CD binding to AR, should provide the basis for the design of new agents to treat androgen-unresponsive prostate cancer.

15. SUBJECT TERMS

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IDEA AWARD

HUMAN MUC1 ONCOPROTEIN IS OF FUNCTIONAL IMPORTANCE TO THE DEVELOPMENT OF PROSTATE CANCER

INVESTIGATOR: Donald W. Kufe, M.D.

INTRODUCTION: Gene expression profiling has demonstrated that the MUC1 oncoprotein is overexpressed in prostate cancers with aggressive clinicopathologic features and is associated with an elevated risk of recurrence [1, 2]. Our hypothesis is that MUC1 contributes to the development of aggressive prostate cancer by directly interacting with the androgen receptor (AR), an important regulator of prostate cancer cell growth. The Specific Aims are: 1) To define the interactions between MUC1 and AR in vitro and in prostate cancer cells (Months 1-18); 2) To assess the effects of MUC1 on AR-mediated gene expression (Months 12-30); and 3) To define the effects of MUC1 on growth and survival of prostate cancer cells in response to androgen stimulation and treatment with antiandrogens (Months 24-36).

BODY: Progress (Months 1-12).

Task 1: To define the interactions between MUC1 and AR in vitro and in prostate cancer cells.

a. Perform studies on the association between endogenous MUC1 and AR in the absence and presence of androgen stimulation (Months 1-6).

Studies have been performed on human CWR22Rv1 prostate cancer cells, which express endogenous MUC1 and a mutant (H874Y) AR. The CWR22Rv1 cells exhibit androgen-independent proliferation and androgen responsiveness. The results demonstrate that the MUC1 C-terminal subunit (MUC1-C) forms complexes with AR constitutively and that this association is increased by dihyrotestosterone (DHT) stimulation (Fig. 1).

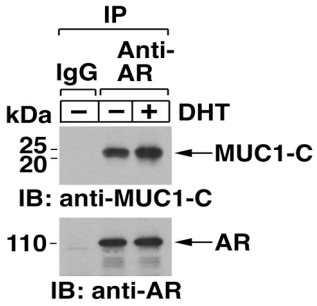


Fig. 1. *Association of MUC1-C and AR in CWR22Rv1 cells. Lysates from CWR22Rv1 cells left untreated or stimulated with DHT for 3 h were immunoprecipitated with anti-AR or a control IgG. The precipitates were immunoblotted with anti-MUC1-C and anti-AR.*

Studies were also performed on the androgen-responsive (LNCaP) and –unresponsive (PC3, DU145) prostate cancer cells. LNCaP cells had undetectable MUC1-C levels (Fig. 2A). By contrast, MUC1-C expression was detectable in both DU145 and PC3 cells (Fig. 2A). PC3 cells have been stably transfected to express an empty vector (PC3-neo) or one expressing the human AR coding region (PC3-AR). AR expression in PC3 cells confers androgen-responsiveness [3] and is associated with downregulation of MUC1-C levels (Fig. 2B).

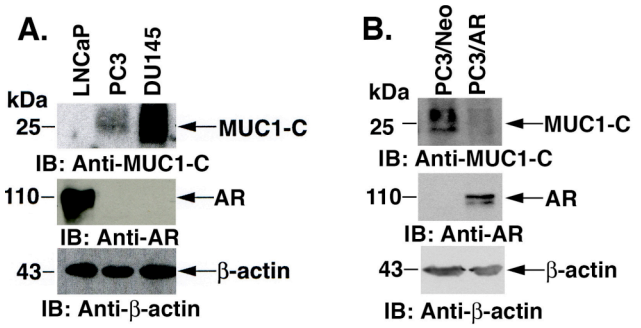
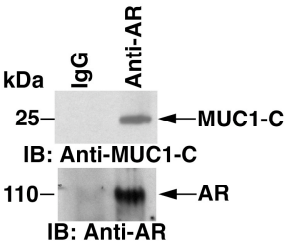


Fig. 2. Expression of MUC1-C and AR in androgen-responsive and –unresponsive prostate cancer cells. *A. Lysates from LNCaP, PC3 and DU145 cells were immunoblotted with the indicated antibodies. B. Lysates from PC3-neo and PC3-AR cells were imunoblotted with the indicated antibodies.*

Coimmunoprecipitation studies using DU145 cell lysates further supported the association of AR and the MUC1-C receptor (Fig. 3).

Fig. 3. *Association of MUC1-C and AR in DU145 cells. Lysates from DU145 cells were immunoprecipitated with anti-AR or a control IgG. The precipitates were immunoblotted with anti-MUC1-C and anti-AR.*



These findings collectively demonstrate that MUC1-C is overexpressed in androgen-unresponsive prostate cancer cells and associates with AR.

b. Generate deletion mutants of AR for direct binding studies with the MUC1 cytoplasmic domain (Months 1-6).

To determine whether MUC1 interacts directly with AR, we prepared vectors expressing the MUC1 cytoplasmic domain (MUC1-CD) and AR (Fig. 4). The MUC1-CD (72 amino acids) is a substrate for c-Src and GSK3β, and contains a binding motif for the Wnt pathway effector, β-catenin [4-7]. MUC1-CD also contains a CQC motif that is necessary for the formation of oligomers [8]. AR is a protein of 919 amino acids that contains a centrally located DNA binding domain (DBD) with two zinc containing modules [9].

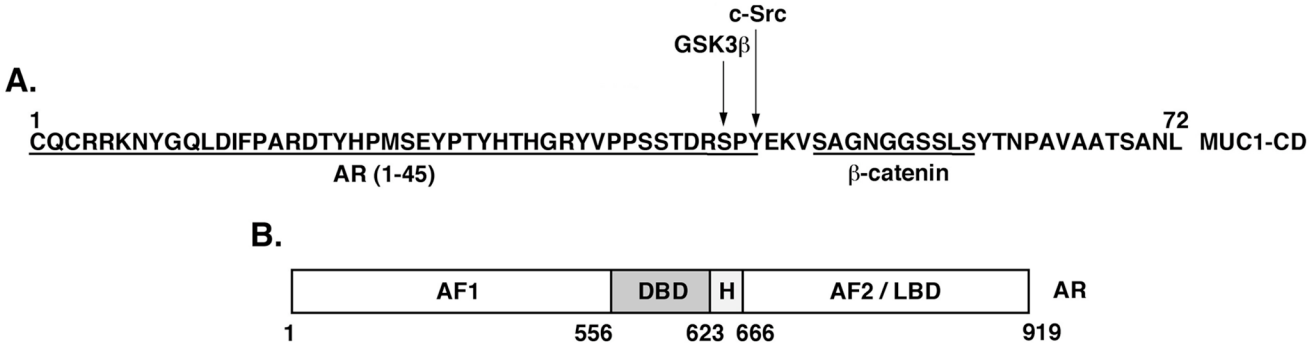


Fig. 4. Structures of MUC1-CD and AR. *A. Amino acid sequence of the 72 amino acid MUC1 cytoplasmic domain. The GSK3β and c-Src phosphorylation sites and the β-catenin binding site are highlighted. B. Schematic representation of the AR AF1 domain, DNA-binding domain (DBD), hinge region (H) and AF2/ligand binding domain (LBD).*

The results of binding studies show that full-length MUC1-CD (amino acids 1-72) and MUC1-CD(1-46), but not MUC1-CD(46-72), interacts with full-length AR (Fig. 5A). In addition, MUC1-CD binds directly to the AR DNA-binding domain (DBD) and not AF1 or AF2 (Fig. 5B).

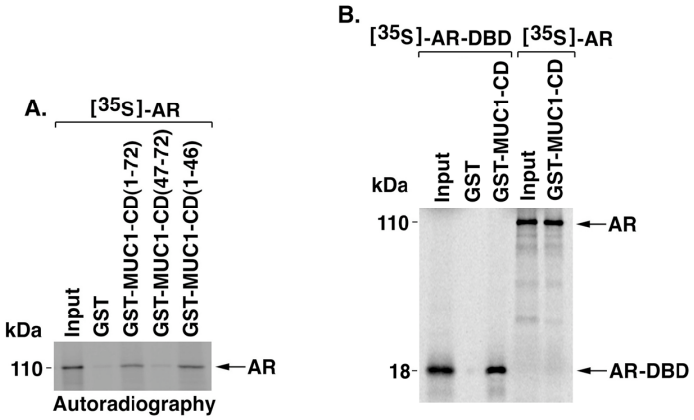


Fig. 5. MUC1-CD binds directly to AR. *A. GST, GST-MUC1-CD and the indicated GST-MUC1-CD deletion mutants were bound to glutathione agarose and incubated with [^{35}S]-labeled AR. B. GST and GST-MUC1-CD were incubated with [^{35}S]-labeled AR or AR DBD deletion mutant. The adsorbates were analyzed by SDS-PAGE and autoradiography.*

These findings demonstrate that the MUC1 cytoplasmic domain binds directly to AR and that the interaction is mediated by MUC1-CD(1-46) and the AR DBD.

c. Generate MUC1 and AR point mutants to more precisely map the sequences involved in the interaction (Months 6-12).

The MUC1-CD contains a CQC motif in the N-terminal region that is involved in the formation of oligomers and thereby the interaction with certain bindings partners [8]. To determine if the MUC1-CD CQC motif confers binding to the AR-DBD, we mutated the cysteines to alanines and performed binding studies with the mutant proteins. Binding of MUC1-CD to the AR-DBD was decreased when mutating CQC to CQA or AQC (Fig. 6). Moreover, binding was abrogated by mutating CQC to AQA (Fig. 6). These findings demonstrate that the MUC1-CD CQC motif confers binding to the AR-DBD.

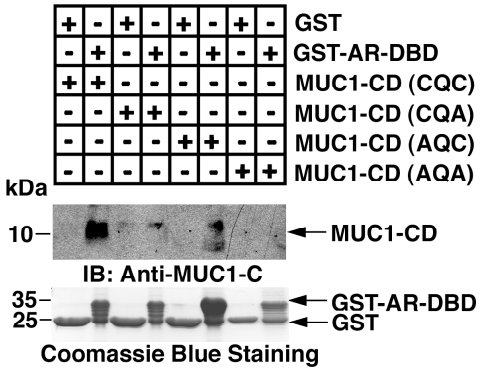


Fig. 6. MUC1-CD CQC motif confers binding to the AR-DBD. *GST and GST-AR-DBD bound to glutathione beads were incubated with the indicated purified wild-type (CQC) and mutant MUC1-CD proteins. The adsorbates were immunoblotted with anti-MUC1-C. Input of the GST proteins was assessed by Coomassie blue staining.*

c-1. Disruption of MUC1-C oligomer formation and binding to AR. The CQC motif in the MUC1 cytoplasmic domain is necessary for the formation of oligomers [8]. Based on the findings that the CQC motif is also necessary for the interaction between MUC1-C and AR, we synthesized a peptide derived from the N-terminal region of MUC1-CD that contains the CQC motif (GO-201; Fig. 7A). A poly D-arginine transduction domain was included in the synthesis to facilitate entry of the peptide into cells (Fig. 7A). As a control, a similar peptide was synthesized in which the CQC motif was altered to AQA (CP-1; Fig. 7A). To assess

binding of the peptides to MUC1-CD, we immobilized His-tagged MUC1-CD to a BIAcore sensor chip. GO-201 bound to His-MUC1-CD with a dissociation constant (Kd) of 30 nM, which is similar to that obtained for binding of full-length MUC1-CD dimers. By contrast, there was no apparent binding of CP-1 (data not shown). Purified His-tagged MUC1-CD forms oligomers as detected by electrophoresis in polyacrylamide gels (Fig. 7B). Incubation of His-MUC1-CD with GO-201 substantially decreased oligomer formation with an increase in monomers (Fig. 7B). Moreover, incubation with CP-1 had little effect (Fig. 7C). To assess effects on MUC1 oligomerization in vivo, 293 cells were transfected with vectors expressing GFP-MUC1-CD and Flag-MUC1-CD (Fig. 7C, left). Complexes of GFP-MUC1-CD and Flag-MUC1-CD were detectable by coprecipitation of lysates from cells not exposed to GO-201 (Fig. 7C, right). In concert with the in vitro results, incubation of the transfected 293 cells with GO-201 was associated with disruption of the interaction between Flag-MUC1-CD and GFP-MUC1-CD (Fig. 7C, right). In addition, CP-1 had no apparent effect (Fig. 7C, right). These results indicate that GO-201 binds to MUC1-CD and blocks formation of MUC1-CD oligomers in vitro and in cells. Studies performed on DU145 prostate cancer cells further demonstrated that treatment with GO-201, and not CP-1, blocks the interaction between AR and MUC1-C (Fig. 7D). These findings are in concert with the demonstration that MUC1-C oligomerization is necessary for direct binding with AR.

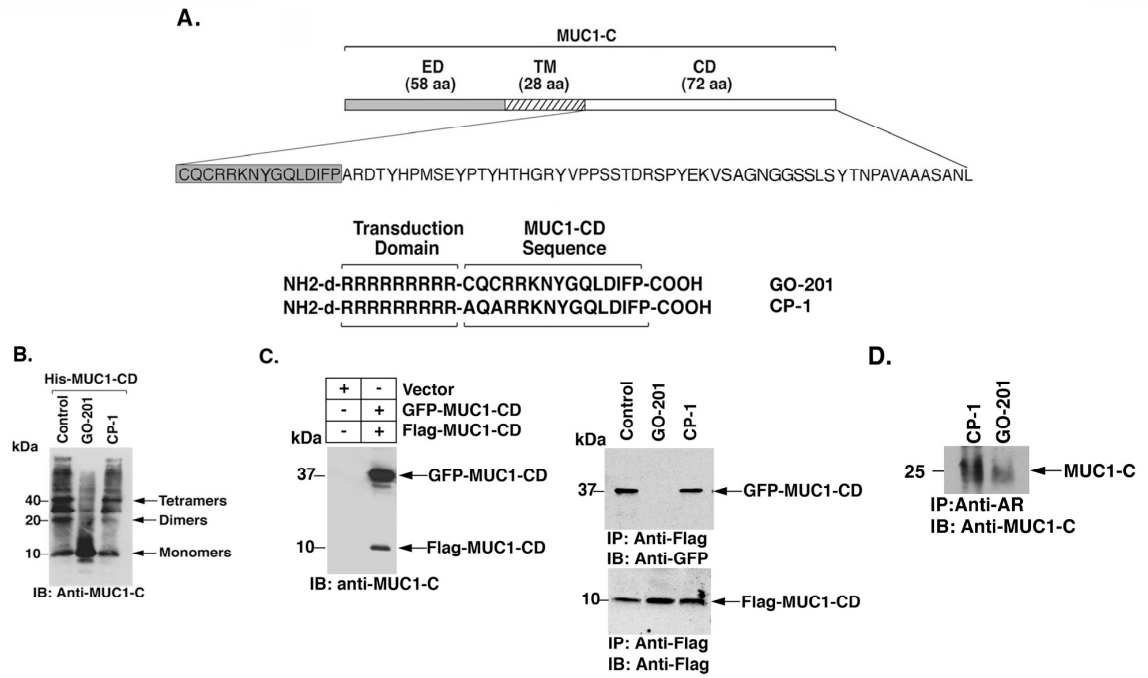


Fig. 7. GO-201 blocks MUC1 oligomerization. *A. Schematic representation of the MUC1-C subunit and the 72 amino acid sequence of MUC1-CD are shown. The N-terminal 15 amino acid (shaded sequence) GO-201 and mutated CP-1 peptides were synthesized with the poly-dArg transduction domain. B. Purified His-MUC1-CD (1.5 mg/ml) was incubated with PBS (Control), GO-201 or CP-1 for 1 h at room temperature. The proteins were separated in a non-reducing SDS-polyacrylamide gel and analyzed by immunoblotting with anti-MUC1-C. C. 293 cells were transiently transfected to express an empty vector or GFP-MUC1-CD and Flag-MUC1-CD. At 48 h after transfection, the cells were left untreated (Control), and treated with 5 μM GO-201 or CP-1 each day for 3 d. The cells were then harvested for immunoblotting with anti-MUC1-C (left panel). Whole cell lysates were also precipitated with anti-Flag and the precipitates were immunoblotted with the indicated antibodies (right panels). D. DU145 cells were treated with 5 μM GO-201 or CP-1 each day for 3 d. Anti-AR immunoprecipitates were blotted with anti-MUC1-C.*

c-2. GO-201 blocks growth and induces necrosis of androgen-unresponsive prostate cancer cells. DU145 prostate cancer cells were treated with 5 μM GO-201 or CP-1. Analysis of DU145 cell number by trypan blue

exclusion staining demonstrated that, compared to untreated cells, the control CP-1 has little if any effect (Fig. 8). By contrast, treatment with GO-201 was associated with slowing of growth and then cell death by day 6 (Fig. 8). Analysis of the cells on day 4 showed that GO-201 treatment is associated with S phase arrest and necrotic cell death (data not shown). Similar results were obtained with PC3 cells. Moreover, GO-201 had no effect on androgen-responsive LNCaP and PC3-AR cells, indicating that GO-201 is selective for androgen-unresponsive prostate cancer cells that express MUC1.

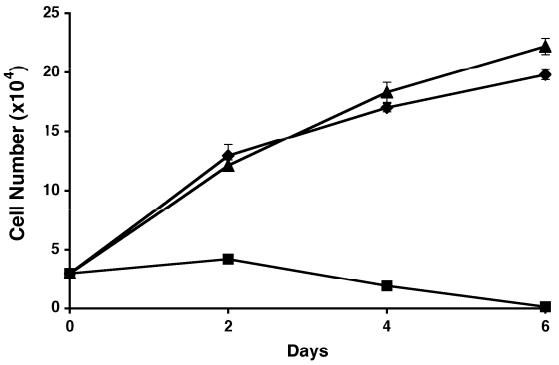


Fig. 8. Effects of GO-201 on DU145 growth and death. *DU145 cells were left untreated (diamonds), and treated with 5 μM GO-201 (squares) or CP-1 (triangles) for the indicated times. Viable cell number was determined by trypan blue staining.*

Based on these findings, we have initiated studies to assess the anti-tumor activity of GO-201 against DU145 and PC3 prostate tumors growing in nude mice. These studies were not proposed in the Tasks funded by this grant. We are therefore conducting these studies with support from other sources.

d. Define the structural basis for the MUC1-AR interaction by cocrystallization and x-ray diffraction (Months 9-18).

We performed 18 crystallization trials for MUC1-CD(1-72), MUC1-CD(1-45) and MUC1-CD(46-72) using different buffer conditions. These trials were performed at 6 mg/ml protein in ProPlex E5 (0.1 M Tris-HCl, pH 8.0, 8% PEG 8,000), F5 (0.1 M Tris-HCl, pH 8.0, PEG 20,000, 0.1 M NaCl) and E10 (0.1 M bicine, pH 9.0, 10% PEG 6,000) buffer conditions. Tiny crystals obtained under these conditions were cryo-protected with 20% glycerol, harvested and screened for diffraction using an Advanced Photon Source (APS) synchrotron. Although some of the crystals were salt, others were protein crystals. However, none of the protein crystals diffracted to high resolution. We will continue attempts at crystallization of MUC1-CD over the next 6 months. However, considering the lack of success thus far and the importance in obtaining MUC1-CD structural information, particularly around the CQC motif, we are entering into a collaboration with investigators who have expertise defining protein structure with NMR and computer simulation modeling.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that MUC1 is predominantly expressed in androgen-unresponsive prostate cancer.
- Demonstration that the MUC1-C receptor subunit associates with the androgen receptor (AR).
- Demonstration that the MUC1-C cytoplasmic domain (CQC motif) binds directly to the AR DNA binding domain.
- Demonstration that peptide drugs which disrupt function of MUC1-C at the CQC motif are effective in inducing death of androgen-unresponsive prostate cancer cells.

REPORTABLE OUTCOMES:

A manuscript is in preparation reporting that disruption of the MUC1-C cytoplasmic domain with a peptide drug is effective in the treatment of androgen-unresponsive prostate cancer cells.

CONCLUSION:

Based on the work performed thus far, our conclusion is that MUC1 is of functional importance to the development of human prostate cancer. The finding that MUC1-C interacts directly with the AR lends support for this conclusion. Moreover, our finding that disruption of the interaction between MUC1-C and AR with GO-201 is effective in inducing death of androgen-unresponsive prostate cancer cells provides further evidence that MUC1 is necessary for their survival. The MUC1 peptide drug thus represents a lead product that could be used to design small molecules for the treatment of hormone-refractory prostate cancer. Tasks planned for the next year should provide additional insights into how our understanding of MUC1 can be exploited for the development of these agents.

REFERENCES:

1. Lapointe, J., C. Li, J.P. Higgins, M. van de Rijn, E. Bair, K. Montgomery, M. Ferrari, L. Egevad, W. Rayford, U. Bergerheim, P. Ekman, A.M. DeMarzo, R. Tibshirani, D. Botstein, P.O. Brown, J.D. Brooks, and J.R. Pollack, Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc. Natl. Acad. Sci. USA, 2004. 101(3): p. 811-6.
2. Cozzi, P.J., J. Wang, W. Delprado, A.C. Perkins, B.J. Allen, P.J. Russell, and Y. Li, MUC1, MUC2, MUC4, MUC5AC and MUC6 expression in the progression of prostate cancer. Clin. Exp. Metastasis, 2005. 22(7): p. 565-73.
3. Cha, T., L. Qiu, C. Chen, Y. Wen, and M. Hung, Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. Cancer Res., 2005. 65(6): p. 2257-2295.
4. Yamamoto, M., A. Bharti, Y. Li, and D. Kufe, Interaction of the DF3/MUC1 breast carcinoma-associated antigen and β-catenin in cell adhesion. J. Biol. Chem., 1997. 272: p. 12492-12494.
5. Li, Y., A. Bharti, D. Chen, J. Gong, and D. Kufe, Interaction of glycogen synthase kinase 3β with the DF3/MUC1 carcinoma-associated antigen and β-catenin. Mol. Cell. Biol., 1998. 18: p. 7216-7224.
6. Li, Y., H. Kuwahara, J. Ren, G. Wen, and D. Kufe, The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3β and β-catenin. J. Biol. Chem., 2001. 276: p. 6061-6064.
7. Huang, L., D. Chen, D. Liu, L. Yin, S. Kharbanda, and D. Kufe, MUC1 oncoprotein blocks GSK3β-mediated phosphorylation and degradation of β-catenin. Cancer Res., 2005. 65: p. 10413-10422.
8. Leng, Y., C. Cao, J. Ren, L. Huang, D. Chen, M. Ito, and D. Kufe, Nuclear import of the MUC1-C oncoprotein is mediated by nucleoporin Nup62. J. Biol. Chem., 2007. 282(27): p. 19321-30.
9. Claessens, F., S. Denayer, N. Van Tilborgh, S. Kerkhofs, C. Helsen, and A. Haelens, Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal., 2008. 6: p. e008.