Award Number:  DAMD17-02-1-0135

TITLE:  High Throughput Synthesis and Screening for Agents Inhibiting Androgen Receptor Mediated Gene Transcription

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REPORT DATE:  February 2003

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release;
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**REPORT DOCUMENTATION PAGE**

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<th>2. REPORT DATE</th>
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<td></td>
<td>February 2003</td>
<td>Annual (1 Feb 02 – 31 Jan 03)</td>
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**4. TITLE AND SUBTITLE**

High Throughput Synthesis and Screening for Agents Inhibiting Androgen Receptor Mediated Gene Transcription

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract (Maximum 200 words)**

Details of the year 1 progress on our grant to define a new treatment of therapeutic resistant prostate cancer is provided. This entails the high throughput synthesis of DNA binding agents related to distamycin, their screening for binding to androgen response elements using a new high throughput DNA binding screen we introduced, and the evaluation for inhibiting androgen receptor mediated gene transcription and the cell proliferation of a prostate cancer cell line.

**14. SUBJECT TERMS:**

prostate cancer, DNA minor groove binding agents, androgen receptor, androgen response elements

**15. NUMBER OF PAGES**

10

**16. PRICE CODE**

**17. SECURITY CLASSIFICATION OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION OF ABSTRACT**

Unclassified

**20. LIMITATION OF ABSTRACT**

Unlimited

NSN 7540-01-280-5500
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Introduction. The objective of our work is the high throughput synthesis of DNA minor groove binding agents based on the distamycin structure and their screening for inhibition of androgen receptor mediated gene transcription, which is unregulated in chemotherapeutic resistant prostate cancer and responsible for disease progression. It represents a novel and unique new target for the treatment of relapse prostate cancer where prognosis is presently very poor.

Body. The results to date in our studies are presented below and refer to Task 1–Task 4 of our proposals’ statement of work.

Task 1: To synthesize chemical combinatorial libraries and compounds. Two objectives were defined in our proposal. The first was to prepare a small set of modifications to compounds 124 and 128. The initial stage of this work has been completed with the preparation of the new derivatives shown in Figure 1. As anticipated, we have shown that their ability to bind DNA increases as the pH increases (more protonated and thus charged). However, we have not yet found derivatives that enter cells better than 124 and 128 and the new derivatives were not better at inhibiting androgen receptor mediated gene transcription. This work continues with additional new derivatives of 124 and 128.

Figure 1. N-Terminal modifications designed to increase cell permeability.

The second objective of Task 1 was to prepare additional DNA binding libraries to discover new leads. Progress on these studies has been superb. An initial library of 2,640 compounds was prepared (Figure 2a) and continues to undergo evaluation for DNA binding and inhibition of androgen receptor mediated gene transcription. A second generation library (Figure 2b) of 4,394 members is in progress and we have assembled the complete set of dimers that must now be linked to the final subunit (as a mixture of 13) to provide the completed library.

For this purpose, we also surveyed a series of building blocks that could be part of this library and
examined their ability to bind DNA in our assay entailing the displacement of ethidium bromide from hairpin DNA's. The results of this work were published and the paper detailing this work is provided in the appendix\(^1\) (C.R. Woods, N. Faucher, B. Eschgfaller, K.W. Bair, and D. L. Boger, Synthesis and DNA binding properties of saturated distamycin analogues, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2647–2650).

Consequently, our progress on Task 1 has been outstanding.

Task 2: To identify libraries that have antitumor activity for prostate cancer cell lines and have high affinity binding to AREs.

- The cytotoxic assay for the prostate cancer cell line, LNCaP, has been set up and validated.
- The first library of 2,640 compounds are beginning to be tested in this cell line.
- The screening of the first library of 2,640 compounds against the ARE hairpin DNAs has been performed.
- Once completed, the data for the two assays will be correlated to identify active constituents that display activity against the prostate cancer cell line and bind the ARE consensus sequence.

Task 3: Define DNA binding selectivity of identified new leads.

- This work has not yet begun and is waiting for the results above, although the assay is available since it was developed by our group prior to submission of this grant (Boger, et al. *J. Am. Chem. Soc.* **2001**, *123*, 5878–5891).\(^2\)

Task 4: Determine the inhibitory effect on AR mediated gene transcription and transactivation.

- As indicated in the original proposal, work on this task will be conducted at the final stages of the grant.

**Key Research Accomplishments.**

- Development of a novel solution-phase approach to the preparation of libraries of sequence selective DNA binding compounds (high throughput synthesis).\(^3\)
- Development of a novel high throughput screen for establishing DNA binding selectivity or affinity. This includes the introduction of the first high throughput screen for a defined DNA sequence (i.e., androgen response element) that can control aberrant gene transcription.\(^2\)
- Definition of previously unexamined features of distamycin responsible for its DNA binding affinity (publication 1).\(^1\)

**Reportable Outcomes.**

**Publications**


**Conclusions.** The work is progressing well and requires no changes in future work. The importance of the work includes not only the potential development of a treatment for relapse (resistant) prostate cancer, but it defines a new approach to treating diseases arising from aberrant gene transcription and, importantly, provides the first scientific tools to approach this problem (high throughput synthesis and screening technology for DNA binding compounds).
References.


Appendix. Attached
Synthesis and DNA Binding Properties of Saturated Distamycin Analogues

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Received 8 April 2002; accepted 28 May 2002

Abstract—A series of saturated heterocyclic analogues of distamycin were prepared and examined. A fluorescent intercalator displacement (FID) assay conducted on p[dA]−p[dT] DNA to obtain C50 values and a hairpin deoxysygonucleotide containing an A/T-rich binding site was used to evaluate DNA binding affinity. It is observed that saturated heterocycles greatly reduce the DNA binding relative to distamycin. © 2002 Elsevier Science Ltd. All rights reserved.

Polyamides composed of N-methylpyrrole (Py), N-methylimidazole (Im), and a growing set of structural analogues bind in the DNA minor groove with predictable sequence selectivity and high affinities. Several factors contribute to the success of polyamide binding including hydrogen bonding, curvature, Van der Waals contacts, and charged end groups. Each component has been explored through the preparation of analogous systems designed to probe the magnitude, requirements, and relative importance of each feature. Yet, the binding effectiveness for polyamides lacking the π-system of the integral heterocaromatic rings has been relatively unexplored. Herein we report the synthesis and evaluation of a series of saturated cyclic polyamides 1–5 (Fig. 1), based on the known, effective DNA minor groove binding agent distamycin.a,b Saturation removes the π-system, alters the conformation, and increases the thickness of the compound, yet maintains the relative structure of distamycin and was used to assess the importance of the π-system. A fluorescent intercalator displacement (FID) assay conducted on p[dA]−p[dT] DNA to obtain C50 values and a hairpin deoxysygonucleotide containing an A/T-rich binding site was used to evaluate DNA their binding affinity.

A solution-phase synthesis of the polyamides was conducted using a series of 1-(3-dimethylaminopropyl)3-

ethylcarbodiimide hydrochloride (EDCI) mediated coupling reactions as described previously where workup, isolation, and purification could be addressed principally by liquid–liquid acid–base extractions. Each series of analogues (1–5) are discussed separately below.

N-CBz pyrrolidine based system (1a and b). N-Boc deprotection of 6a (HCl–Et2OAc) provided the amine 7 as the hydrochloride salt and methyl ester hydrolysis of 6b (LiOH, THF–MeOH–H2O) provided carboxylic acid 8 (Scheme 1).

After deprotection, 7 and 8 were coupled to produce dimer 9a (EDCI, HOBt, DMF, 25 °C, 13–20 h, 66%)

Figure 1. Distamycin and saturated heterocyclic polyamides 1–5.
before efficient conditions were found (H₂, 10% Pd/C, 10 equiv TFA, MeOH, 25°C, 4 h, entry 6). Thus, although N-CBz deprotection was slow and typically problematic, acid catalysis with inclusion of TFA provided 2 in good conversions.

**Furan based system (3a and b).** Transformation of the monomer 12 and its enantiomer into their respective functionalized trimers 3a and 12b was performed using the strategy detailed above for 1a and b (Schemes 1 and 2). However, the tetrahydrofuran-based compounds proved highly water soluble. Thus, removal of excess starting materials, reagents, and side products by simple acid-base extraction was not possible and chromatographic purifications were required.

**Cyclopentane based system (4a).** Transformation of the monomer 18 into 4a was performed uneventfully using the strategy detailed above (Schemes 1 and 2).

N-Me pyrrolidine based system (5a). N-Boc deprotection (HCl-dioxane) of 24 provided the amine 25 as the hydrochloride salt and methyl ester hydrolysis of 24 (LiOH, THF-MeOH) provided the lithium carboxylate 26 (Scheme 3).

Following the strategy detailed above, the lithium salt 26 was used to provide trimer 28 (Scheme 4). However, coupling of the carboxylic acid derived from 28 with 3-aminopropionitrile afforded 29 in only moderate yield.

---

### Table 1. Hydrogenolysis conditions for 1a using 10% Pd/C

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H₂, MeOH, 25°C, 24-48 h</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>H₂, MeOH, 2N HCl, 25°C, 24 h</td>
<td>Decomposition</td>
</tr>
<tr>
<td>3</td>
<td>4% HCOOH, MeOH, 25°C, 14 h</td>
<td>Decomposition</td>
</tr>
<tr>
<td>4</td>
<td>H₂, acetone-MeOH, 25°C, 24 h</td>
<td>No reaction</td>
</tr>
<tr>
<td>5</td>
<td>H₂, 2 equiv HOAc, MeOH, 25°C, 24 h</td>
<td>No reaction</td>
</tr>
<tr>
<td>6</td>
<td>H₂, 10 equiv TFA, MeOH, 25°C, 4 h</td>
<td>2a, 64%</td>
</tr>
</tbody>
</table>

### Table 2. C₅₀ values

<table>
<thead>
<tr>
<th>Compd</th>
<th>C₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distamycin</td>
<td>0.46</td>
</tr>
<tr>
<td>1a</td>
<td>4.70</td>
</tr>
<tr>
<td>1b</td>
<td>5.10</td>
</tr>
<tr>
<td>2a</td>
<td>6.37</td>
</tr>
<tr>
<td>2b</td>
<td>8.55</td>
</tr>
<tr>
<td>3a</td>
<td>5.40</td>
</tr>
<tr>
<td>3b</td>
<td>5.50</td>
</tr>
<tr>
<td>4a</td>
<td>7.91</td>
</tr>
<tr>
<td>5a</td>
<td>6.70</td>
</tr>
</tbody>
</table>

p[dA]-p[dT] 8.8 µM bp, EtBr 4.4 µM.
(20%, Scheme 2). Consequently, an alternative convergent synthesis was used to provide 29 (Scheme 4). Coupling 26 with 3-aminopropionitrile (HATU, i-Pr₂NEt, DMF, 25°C, 80%) yielded nitrile monomer 30, which was subsequently N-Boc deprotected (HCl-EtOAc) and coupled with the carboxylic acid derived from 27 (LiOH, THF-MeOH-H₂O) using standard coupling conditions (HATU, i-Pr₂NEt, DMF, 25°C, 60%) to provide 29 in a more satisfactory yield. Treatment of 29 with freshly prepared 8N HCl-EtOH led to N-Boc deprotection and imidate formation. Subsequent treatment of the imidate with NH₃-EtOH provided the corresponding amine which was formulated using N-formyl imidazole freshly prepared by treating carbonyldiimidazole with formic acid to provide 5a.¹⁴

**Initial assessment of DNA binding.** The compounds 1–5 were initially screened for DNA binding using [dA]–[dT] in a fluorescence intercalator displacement (FID) assay.¹² The relative binding affinities were established by monitoring the loss of fluorescence derived from titration displacement of prebound ethidium bromide from [dA]–[dT]. A comparison of the compound concentration required for 50% displacement of the ethidium bromide (C₅₀) revealed that none of the saturated analogues compared well to distamycin (Table 2). The best of the analogues, N-CBz pyrrolidine 1a, required a 10-fold greater concentration relative to distamycin (4.70 vs 0.46 μM), while the remaining analogues required even higher concentrations (5.10–8.55 μM). Interestingly, one stereochemical configuration, (R)c.-ring(5)N-ring (the a analogues), consistently performed better than their respective enantiomers.

**Binding to a hairpin deoxyoligonucleotide containing an A/T-rich binding site.** In an attempt to assess binding constant comparisons of 1–5, a FID titration assay was performed using a DNA hairpin with an A/T-rich binding site of 5 bp. As above, the analogues exhibited low binding affinity toward the hairpin DNA, yielding flat fluorescence reduction curves too shallow for accurate Scatchard analysis.¹⁶ Qualitatively, their behavior can be compared using the ratio of fluorescence present before (F₁₀₀₀) and after the addition of one equivalent of agent (Fᵢ).¹⁷ Using the Fᵢ/F₁₀₀₀ value (Table 3). With distamycin, a Fᵢ/F₁₀₀₀ of 0.48 is observed, whereas much higher values are observed with the saturated systems (0.77–9.2). The trends for hairpin and [dA]–[dT] DNA binding (Fᵢ/F₁₀₀₀ versus C₅₀) are similar with the N-CBz derivatives (1a and b) exhibiting the better binding properties than the remaining analogues. However, additional insights are difficult to extrapolate due to the low binding ability.

**Discussion**

One major impact of saturating the distamycin pyrrole rings with 2 or 5 is the introduction of three additional basic nitrogens. Those of 2 are present as secondary amines while those of 5 are tertiary amines. Although not observed, one might have suspected that pH 7 protonation of these amines might provide positively charged centers that would benefit from stabilizing electrostatic interactions with DNA enhancing their affinity. In addition, the comparable behavior of 2 and 5 with either 3 or 4, suggest that the presence of the basic amines do not enhance or inhibit binding relative to the impact of introduction of the saturated versus unsaturated

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**Table 3.** Fᵢ/F₁₀₀₀ values of distamycin agents 1–5 with hairpin DNA

<table>
<thead>
<tr>
<th>Compd</th>
<th>Fᵢ/F₁₀₀₀*</th>
<th>Curve description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distamycin</td>
<td>0.48</td>
<td>Good</td>
</tr>
<tr>
<td>1a</td>
<td>0.88</td>
<td>Flat</td>
</tr>
<tr>
<td>1b</td>
<td>0.77</td>
<td>Flat</td>
</tr>
<tr>
<td>2a</td>
<td>0.90</td>
<td>Flat</td>
</tr>
<tr>
<td>2b</td>
<td>0.88</td>
<td>Flat</td>
</tr>
<tr>
<td>3a</td>
<td>0.90</td>
<td>Flat</td>
</tr>
<tr>
<td>4b</td>
<td>0.90</td>
<td>Flat</td>
</tr>
<tr>
<td>5a</td>
<td>0.92</td>
<td>Flat</td>
</tr>
<tr>
<td>5b</td>
<td>0.83</td>
<td>Flat</td>
</tr>
</tbody>
</table>

*Fᵢ/F₁₀₀₀ is the ratio of fluorescence before (F₁₀₀₀) and after the addition of 1 equivalent of agent (Fᵢ).
heterocycles. Interestingly, the most effective analogue in the series is 1 bearing the hydrophobic N-CBz protecting group. However, even 1 would not be considered an effective DNA binding agent. Finally, the all carbon cyclopropyl-based analogue 4 emerged in the DNA binding assays as the poorest analogue in the series.

Although several features imparted by the structural changes could be responsible for the poor binding of 1–5, the magnitude of the reductions is notable. From molecular modeling studies, a minor groove complementary shape, hydrogen bonding, and stabilizing electrostatic interactions analogous to those observed with distamycin are possible for 1–5 and the hydrophobic character covered by the series brackets that of distamycin. Nonetheless, no saturated analogue in the series came close to the DNA binding affinity observed with distamycin illustrating the importance of the unsaturated heterocycles.18

Acknowledgements

We gratefully acknowledge the financial support of the National Institutes of Health (CA41986, CA78045), Novartis, and the Department of Army (DAMD17–02–1-0135).

References and Notes

4. Experimental details and characterization for intermediates and final products can be provided upon request.
5. For 9a, 1H NMR (acetone-d6, 400 MHz) δ 7.66 (m, 1H), 7.40–7.30 (m, 10H), 6.34 (bs, 1H), 5.13–4.95 (m, 4H), 4.47–4.24 (m, 4H), 3.79 and 3.39 (s, 3H), 3.75 and 3.38 (m, 4H), 2.20 (m, 4H), 1.38 (s, 9H); IR (NaCl, film) umax 3315, 2975, 1700, 1644, 1531, 1417, 1346, 1169 cm−1; MALDI–HRMS (DHB) m/z 647.2686 (M+Na+), C32H36N4O3 requires 647.2687; [α]D20 +23 (c 1, THF). For 9b, [α]D20 +22 (c 1, THF).
6. For 10a, 1H NMR (acetone-d6, 400 MHz) δ 7.93 (m, 2H), 7.35–7.29 (m, 15H), 6.39 (m, 1H), 5.11–4.95 (m, 6H), 4.49–4.23 (m, 6H), 3.72 and 3.60 (s, 3H), 3.72 and 3.37 (m, 6H), 2.18 (m, 6H), 1.38 (s, 9H); IR (NaCl, film) umax 3311, 2933, 1704, 1537, 1420, 1356, 1169 cm−1; MALDI–HRMS (DHB) m/z 893.3688 (M+Na+), C40H43N4O12 requires 893.3692; [α]D20 +24 (c 1, THF). For 10b, [α]D20 −17 (c 1, THF).
7. For 11a, 1H NMR (acetone-d6, 400 MHz) δ 7.76 (m, 1H), 7.58 (m, 2H), 7.35–7.30 (m, 15H), 6.32 (bs, 1H), 5.14–4.92 (m, 6H), 4.4–4.3 (m, 6H), 3.73 and 3.42 (m, 8H), 2.54 and 2.56 (m, 2H), 1.20 (m, 6H), 1.38 (s, 9H); IR (NaCl, film) umax 3309, 2964, 2343, 1604, 1539, 1420, 1357, 1169 cm−1; MALDI–HRMS (DHB) m/z 931.3929 (M+Na+), C42H45N5O3 requires 931.3961; [α]D20 +29 (c 0.1, THF). For 11b, [α]D20 −29 (c 0.1, THF).
8. For 1a, 1H NMR (methanol-d4, 400 MHz) δ 8.31 (brs, 2H), 8.02 (brs, 1H), 7.33 (m, 15H), 5.12–4.88 (m, 6H), 4.5–4.3 (m, 6H), 3.81 (m, 3H), 3.43 (m, 3H), 2.67 (m, 2H), 2.19 (m, 6H); IR (NaCl, film) umax 3243, 1662, 1542, 1542, 1356, 1126 cm−1; MALDI–HRMS (DHB) m/z 854.3834 (M+H+), C39H37N4O10 requires 854.3831; [α]D20 +26 (c 0.1, MeOH).
9. For 3a, 1H NMR (methanol-d4, 500 MHz) δ 8.02 (s, 1H), 4.47 (m, 6H), 4.11 (m, 3H), 3.76 (m, 3H), 3.47 (m, 2H), 2.69 (m, 2H), 2.31 (m, 6H); IR (NaCl, film) umax 3128, 2851, 1668, 1630, 1085 cm−1; MALDI–HRMS (DHB) m/z 455.2244 (M+H+), C19H30N4O2 requires 455.2249; [α]D20 −25 (c 0.1, MeOH). For 3b, [α]D20 +22 (c 0.1, MeOH).
13. The lithium salt 26 was used instead of the carboxylic acid to avoid potential coupling problems with the zwitterionic form of the amino acid.
14. For 5a, 1H NMR (methanol-d4, 400 MHz) δ 8.01 (s, 1H), 4.37 and 3.99 (m, 6H), 3.56 and 3.43 (m, 3H), 3.18 (m, 3H), 2.81 and 2.68 (m, 2H), 2.40 (m, 11H), 2.14 (m, 6H); IR (NaCl, film) umax 2939, 2852, 1643, 1639, 1580, 1256, 1107 cm−1; MALDI–HRMS (DHB) m/z 516.3022 (M+Na+), C39H37N4O4 requires 516.3017; [α]D20 +88 (c 0.1, MeOH).
17. Addition of one equivalent of agent from the titration curves was chosen arbitrarily for the comparisons.
18. 1a, 1b, 3a, and 3b were inactive in an L1210 cytotoxic assay (IC50 > 10 μM).