ORIGINAL ARTICLE
Pharmacodynamic study of disulfiram in men with non-metastatic recurrent prostate cancer

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BACKGROUND: Preclinical drug screens identified disulfiram as a potent in vitro inhibitor of prostate cancer (PCA) cell growth. Although many mechanisms for its anticancer activity have been proposed, tumor suppressor gene re-expression through promoter demethylation emerged as one of the more plausible.

METHODS: We conducted an open-label, dose escalation trial of disulfiram in men with non-metastatic recurrent PCAs after local therapy. Dose escalation occurred if a demethylating 'response' (that is, ≥10% decrease in peripheral blood mononuclear cell (PBMC) global 5-methyl cytosine (5meC) content) was observed in ≤3 patients in cohort 1. Cohorts 1 and 2 received disulfiram 250 mg and 500 mg daily, respectively. The primary end point was the proportion of subjects with a demethylation response. Secondary end points included the rate of PSA progression at 6 months, changes in PSA doubling time and safety/tolerability.

RESULTS: Changes in global 5meC content were observed in two of nine patients (22.2%) in cohort 1 and 3 of 10 (30.0%) in cohort 2. Only five subjects were on trial for ≥6 months, all were in cohort 1 and all had PSA progression by 6 months. No changes in PSA kinetics were observed in either cohort. Disulfiram was poorly tolerated with six patients experiencing grade 3 adverse events (three per cohort). Three of the responders displayed pretreatment instability in their 5meC content.

CONCLUSIONS: A minority of patients had transient global PBMC demethylation changes. Instability in 5meC may limit the reproducibility of these findings, limiting our ability to confirm our hypothesis. Given the toxicities and no clinical benefits, further development of disulfiram should not be pursued in this population.

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INTRODUCTION
Epigenetic changes in prostate cancer (PCA) are recognized as occurring at the earliest phase of carcinogenic transformation.1 Further, alterations in the epigenome persist and evolve during invasion, metastasis and progression.2,3,4 One of the most recognized epigenetic alterations, methylation of cytosines in gene promoter regions, can lead to tumor suppressor gene silencing and in turn contribute to the cancer phenotype.5 DNA methyltransferases (DNMTs) constitute the group of enzymes responsible for maintaining these CpG methylation marks, and have been the primary target of drugs developed as demethylating agents.1 Two nucleoside DNMT inhibitors, azacitidine and decitabine, are currently approved for the treatment of myelodysplastic syndrome. Their extensive incorporation into DNA may in theory lead to increased toxicity and carcinogenesis.6–8

Preclinical compound screens revealed that disulfiram potently inhibits PCA cell line growth.8,9 Given that disulfiram is a known thiol-reactive compound, we hypothesized that it may inhibit DNMTs, which use a catalytic cysteine residue in the methyltransferase reaction.10 Indeed, preclinical work by our group established that disulfiram was able to inhibit DNMT1 activity and could lead to DNA demethylation in PCA cells; manifested as global reductions in 5-methyl cytosine (5meC) content, decreased methylation in APC and RARB gene promoters, and subsequent gene re-expression.8,9 There have been a multitude of additional proposed mechanisms for disulfiram’s antitumor activity, including that the disulfiram analogue pyrrolidine dithiocarbamate may be able to chelate copper, possibly inhibiting proteasomes or exerting an antiangiogenic effect.11–22

To evaluate disulfiram’s potential as an epigenetic therapy, we conducted a translational pilot trial (registration ID: NCT01118741) through the Department of Defense Prostate Cancer Clinical Trials Consortium. Our primary objective was to evaluate whether disulfiram produced demethylating changes in men with biochemically recurrent PCAs through quantifying changes in global 5meC DNA content in peripheral blood mononuclear cells (PBMC).2,3

MATERIALS AND METHODS
Inclusion criteria
Eligible patients were ≥18 years old, previously treated with local therapy (for example, radiation or surgery) for histologically proven prostatic adenocarcinoma and subsequently developed biochemically recurrent disease (confirmed rising PSA of ≥1 ng·mL−1 at least 2 weeks apart). Biochemically recurrent patients were the focus given that this population would be less likely to experience serious morbidity as a result of disease progression should disulfiram lack an anti-PCa effect. Subjects were required to have adequate bone marrow, renal and liver function and no evidence of metastasis. All previous local therapies must have been discontinued at least 4 weeks before enrollment. Patients were allowed to have received prior systemic therapies. Those treated with hormonal...
therapy were allowed to enroll if their treatment was discontinued >6 months prior and had testosterone recovery. Patients were also required to have an Eastern Cooperative Oncology Group performance status of ≤ 2. Participants had to agree not to drink alcohol during the study and for 14 days after its completion. Participants signed an Institutional Review Board-approved consent form.

Treatment plan
This was an open-label, prospective, multicenter, clinical trial evaluating disulfiram in men with biochemically recurrent PCa. Two cohorts were planned with doses selected on the basis of the Food and Drug Administration approved doses for disulfiram. A dose escalation occurred based on whether a demethylating effect (that is, >10% decrease in global 5mC content from PBMC) was observed at the initial dose. Cohorts 1 and 2 received disulfiram 250 mg and 500 mg daily, respectively. If a demethylating response was observed in <3 out of the 9 subjects initially enrolled to cohort 1, then cohort 2 was opened. If there was evidence for a demethylating response in ≥3 subjects in cohort 1 or 2, then that cohort would expand to treat 17 total patients (Figure 1). Toxicity of disulfiram was also taken into account, with dose escalation or cohort expansion only occurring if grades 3–4 adverse events (AEs) were observed in <3 subjects out of the first 9 patients enrolled to cohort 1 or 2. Toxicity was defined using the National Cancer Institute (NCI) guidelines in the Common Terminology Criteria for Adverse Events Version 3.0.

Global methylation assay
Blood samples were drawn serially at the time of screening, on day 1/cycle 1 and weeks 4, 8, 16, and 24. Samples were stored at -4°C or on ice before PBMC DNA extraction. Samples not drawn at the primary site (Johns Hopkins) were shipped overnight to the primary site for processing. DNA samples were batched and stored at -70°C. Global DNA methylation status was determined on PBMC DNA using a high pressure liquid chromatography-tandem mass spectrometry assay as described previously. The overall 5mC content (as percentage of total 2'-deoxycytidine content) in genomic DNA was compared before and after the treatment. Given slight variations in the assay between batches, all batches were normalized to the same mean.

Pharmacokinetics
Disulfiram plasma concentrations were not measured owing to stability issues. Plasma samples were obtained at baseline and before disulfiram administration (minimum concentration (Cmin)) at week 4 to measure the metabolite, S-Methyl, N,N-diethylthiocarbamate (MedDC). Briefly, 0.1 ml plasma was extracted with acetonitrile precipitation. MeDDC separated on a Waters X-Terra MS C18 (50 × 2.1 mm, 3.5 μm; Milford, MA, USA) column with 0.1% formic acid in acetonitrile/2 mM ammonium acetate (70:30, v/v) using an isocratic flow of 0.2 ml min⁻¹ for 5 min. MedDC was quantitated over the range of 10–2000 ng ml⁻¹. As disulfiram was administered in the evening for multiple patients, the reported Cmin was corrected to the actual/theoretical Cmin at 24 h utilizing the following formula:

\[
C_{\text{min, actual}} = C_{\text{min, reported}} \times e^{k \times t}
\]

Where k as the elimination rate constant based on a reported T1/2 of 6.3 h, and t was the difference between the time of the Cmin, reported sample and a 24 h Cmin.24 If the Cmin, sample was below the lower limit of quantitation or calculated to be below the lower limit of quantitation, a concentration difference between replicates was 2.2% (95% confidence interval 0.4–4%). Given that the upper bound of the 95% confidence interval for percent difference between replicates was 4%, a ≥10% decrease in 5mC was felt unlikely to occur by chance.

Secondary end points included PSA progression at 6 months (confirmed PSA >50% above baseline and ≥2 ng ml⁻¹ above nadir), changes in PSA kinetics, safety, pharmacokinetic/pharmacodynamic correlates and ceruloplasmin changes. AEs were assessed by the lead site on an ongoing basis, with quarterly reviews of the clinical data at a minimum. Severe AEs were reviewed as they were reported. These were assessed by the medical monitor and their decision regarding the safety of continuing the study was shared with the investigators. The study could be terminated based on the assessment of either the lead site, study sponsor or the principal investigator.

Statistical analysis
The dose escalation scheme was designed using a Simon two-stage approach (Figure 1). A study cohort of 17 would have yielded 95% power to detect a 60% demethylating response rate compared with a null rate of 20%, with a one-sided alpha error of 0.05. The probability of stopping the trial with only nine patients in either cohort was 2.5% assuming that the true response rate was 60%. The probability of stopping the trial if the true demethylating response rate was 20% in either cohort was 73.8%.

RESULTS
Patients
Between June 2010 and August 2011, a total of 19 patients were screened and enrolled in this study—9 in cohort 1 and 10 in cohort 2. Three patients in cohort 1 received prior hormonal therapy, whereas none in cohort 2 did. Otherwise, baseline characteristics between cohorts were well matched (Table 1).

Demethylating response
Two patients out of 9 (22.2%) in cohort 1 had a demethylation response. Cohort 2 was therefore opened, and 10 patients were accrued at that dose level. Three of the first 9 (33.3%) patients enrolled in cohort 2 were responders, and although this met our threshold for cohort expansion, toxicities at this dose level were, in the opinion of the investigators, too toxic to allow accrual beyond the 10th patient. There were no differences in the demethylating response rate observed between cohorts during any of the treatment cycles. Demethylating responses were generally transient, persisting for one or two cycles only (Table 2).

Toxicity
Although there were no grade 4 AEs, the drug was in general poorly tolerated at the higher dose. Six patients experienced one grade 3 AE (three per cohort) (Table 3). Other common grade 1 and 2 AEs included fatigue, gastrointestinal toxicity, ataxia/dizziness and constitutional symptoms. In contrast to the low-dose cohort where the most common reason for coming off study was PSA or radiographic progression (n = 6), those in the high-dose cohort most often came off study due to toxicity (n = 5). Toxicities leading to patient withdrawal in the high-dose cohort included grade 3 diarrhea (n = 1), grade 2 fatigue (n = 2), grade 2 neuropathy (n = 1) and unspecified toxicity (n = 1). Overall, there was a trend toward fewer median number of completed cycles in the high-dose compared with the low-dose cohort (five vs nine cycles).
Global methylation assay
To assess the stability of global 5\(^{m}\)C DNA content over time, we obtained two serial blood samples before initiating disulfiram. These samples were obtained in all but one patient in cohort 2. The median time between pretreatment samples was 6.5 days (range, 2–76 days), with a median absolute change in 5\(^{m}\)C content of 9.6 months (range, 7.5–15.9 months).

PSA end points
When stratified by dose level, no differences in PSA-based outcomes were observed (Table 4). In addition, there was no difference in PSA-based end points for these individuals was 12.38% occurring after a median of 6 days. These subjects represented the three responders from cohort 2. Six days. These subjects represented the three responders from cohort 2.

| Reason off study, n (%) | Disulfiram, 250 mg | Disulfiram, 500 mg |
|--------------------------|--------------------|--------------------|
| Toxicity                 | 1 (11)             | 5 (50)             |
| PSA progression          | 4 (44)             | 2 (20)             |
| Radiographic progression | 2 (22)             | 0                  |
| Patient decision         | 1 (11)             | 3 (30)             |
| Median number of cycles completed\(^a\) | 9                  | 5                  |

\(\text{Grade 3 or higher AE, n (%)}\)

| Double vision | 1 (11)\(^b\) | 1 (11) |
| Hearing loss   | 1 (11)     |
| LFT abnormality| 1 (11)     |
| Diarrhea       | 1 (10)\(^b\) |
| Constipation   | 1 (10)     |
| Ataxia         | 1 (10)     |

Abbreviations: AE, adverse event; LFT, liver function test.
\(^a\)Difference in median number of completed cycles trended toward statistical significance (P = 0.12).
\(^b\)Resulted in subject coming off study.

Pharmacokinetic/pharmacodynamic
The 12 patients enrolled at the primary study site (Johns Hopkins) had their plasma disulfiram concentrations indirectly measured via its metabolite MeDDC. The MeDDC \(C_{\text{min,actual}}\) was only detectable in two patients, one out of nine at 250 mg and one out of three at 500 mg. MeDDC \(C_{\text{min,actual}}\) was significantly higher for the responders (\(n = 4\)) compared with non-responders (\(n = 7\)) (13.2 and 14.5 mg \(\text{ml}^{-1}\) in two patients vs undetectable, P = 0.05). No other significant correlations with MeDDC \(C_{\text{min,actual}}\) were determined. This data should be interpreted with caution given that MeDDC \(C_{\text{min,actual}}\) was only detectable in a minority of patients.

Ceruloplasmin levels
All of the patients enrolled in the trial had at least two ceruloplasmin levels obtained. None of those enrolled had a significant change in ceruloplasmin level between treatment cycles. Median ceruloplasmin level for the entire cohort remained stable between treatment cycles as well.

CONCLUSIONS
Disulfiram may lead to transient demethylating changes in a subset of patients, with those who achieve higher disulfiram metabolite (MeDDC) levels more often displaying evidence of demethylation. We also found that disulfiram’s use at high-doses was significantly limited due to toxicity. Future studies to develop less toxic non-nucleoside analog DNMT inhibitors may be warranted.

Table 1. Patient demographics

|                        | Disulfiram, 250 mg (N = 9) | Disulfiram, 500 mg (N = 10) | Combined (N = 19) |
|------------------------|---------------------------|-----------------------------|-------------------|
| Age, years (median (min, max)) | 68 (57, 77)               | 62.5 (56, 67)               | 65 (56, 77)       |
| Race (Caucasian, n (%))   | 9 (100%)                  | 9 (90%)                     | 18 (94.7%)        |
| Prior hormonal therapy, n (%) | 3 (33.3%)                | 0 (0%)                      | 3 (15.8%)         |

Characteristics at diagnosis

|                        | Disulfiram, 250 mg (N = 9) | Disulfiram, 500 mg (N = 10) | Combined (N = 19) |
|------------------------|---------------------------|-----------------------------|-------------------|
| Gleason score, median (min, max) | 7 (6, 9)                  | 7.5 (6, 9)                  | 7 (6, 9)          |
| PSA, median (min, max)  | 5.4 (0.2, 28)             | 5.6 (2.2, 20)               | 5.4 (0.2, 28)     |

Note: all prior hormonal therapies were given intermittently for the purpose of palliation.

Table 2. Summary of subjects exhibiting a demethylating response to disulfiram

| Dose (mg) | Number of cycles received | Demethylation response (1 = yes, 0 = no, X = missing) |
|----------|---------------------------|-----------------------------------------------------|
| 500      | 4                         | C2 1 C3 1 C5 X EOS 0                                  |
| 500      | 5                         | C2 0 C3 0 C5 0                                      |
| 500      | 6                         | C2 0 C3 0 C5 X                                      |
| 250      | 2                         | C2 1 C3 X C5 X X                                   |
| 250      | 9                         | C2 0 C3 0 C5 1                                      |

Samples for a given cycle were drawn on day 1 of said cycle. The end of study (EOS) sample was obtained at the time a subject came off study. Missing values were due to subjects coming off trial early.

Table 3. Reason for study discontinuation and toxicity summary.

| Reason for study discontinuation, n (%) | Disulfiram, 250 mg | Disulfiram, 500 mg |
|----------------------------------------|--------------------|--------------------|
| PSA progression                        | 4 (44)             | 0                  |
| Grade 3 or higher AE                    | 1 (11)\(^b\)       | 1 (11)             |
| Toxicity                               | 1 (11)             | 5 (50)             |
| Radiographic progression                | 4 (44)             | 2 (20)             |
| Routine decision                        | 1 (11)             | 3 (30)             |
| Radiation                              | 1 (11)             | 1 (10)             |
| Ceruloplasmin                          | 1 (11)             | 1 (10)             |

Abbreviations: AE, adverse event; LFT, liver function test.
\(^a\)Difference in median number of completed cycles trended toward statistical significance (P = 0.12).
\(^b\)Resulted in subject coming off study.
We evaluated disulfiram’s ability to achieve our proposed target biological effect, global DNA demethylation. By relying on PBMC 5mC content, a translational pharmacodynamic end point that measures global DNA methylation, we hoped to avoid the pitfalls associated with PSA-based end points in the rising PSA window.3,8,23,25 Only transient demethylating changes in a minority of patients were observed, and although the obvious explanation for this lack of a robust response is that disulfiram is a poor DNMT inhibitor, other possibilities exist. For instance, a recent report has shown that although most display very little alteration in their 5mC content over time, a subset of individuals, typically in familial clusters, demonstrate dynamic change in their 5mC content.26 Given that three individuals in this study exhibited >10% changes in 5mC content before receiving disulfiram speaks to the high likelihood that some of the observed demethylating responses may have been a result of intrinsic variability in DNA methylation and not an effect of the study drug. The issue of 5mC content stability in PBMC over time, particularly in men with PCa, as well as the correlation between tumoral and PBMC methylation and the clinical significance of changes in global methylation warrant further study before utilizing 5mC content as an end point in future trials. It is also uncertain whether disulfiram’s parental compound or one of its intermediate metabolites may be a more effective DNMT inhibitor and/or an anticancer agent. Disulfiram’s known complex metabolic processing may explain the differences observed between the promising preclinical data and the modest effects seen in this trial.24

The possibility of disulfiram exerting an antitumor effect through a mechanism other than DNA demethylation was also explored. The copper chelator ATN-224 has demonstrated mixed results in regard to its ability to cause favorable PSA changes in men with PCa, with one out of two trials demonstrating an effect on PSA.27,28 Given that the disulfiram metabolite pyrrolidine dithiocarbamate has been proposed to exert an anticancer effect through copper chelation, we sought to explore this as an alternative mechanism for disulfiram’s preclinical anticancer activity. We did not observe any changes in ceruloplasmin levels in those enrolled on this study, implying that pyrrolidine dithiocarbamate does not impact copper levels. Whether intratumoral copper-pyrrolidine dithiocarbamate complexes were formed was not directly addressed.

No changes in PSA-based outcomes were observed on this trial. The Prostate Cancer Working Group has pointed out that considerable variation in PSA and PSA kinetics can occur during trials evaluating therapeutic interventions in men in the rising PSA clinical state, and have emphasized the need to define intervention-specific PSA-based outcome at the outset of a study.29 In the case of this trial, our predefined PSA-based outcome was the rate of progression at 6 months. Although it is difficult to make definitive statements regarding the effect of disulfiram on PSA given that only five patients remained on trial ≥6 months, the fact that these five subjects all progressed has dampened our enthusiasm for disulfiram in this setting.

Table 4. PSA kinetics summary

| Pretreatment PSA velocity (ng/ml per year), median (range) | Disulfiram, 250 mg (N = 9) | Disulfiram, 500 mg (N = 10) | P |
|----------------------------------------------------------|----------------------------|----------------------------|---|
| Pretreatment PSADT (days), median (range)                | 192.2 (16, 445.8)          | 108.5 (68.1, 402.2)        | 0.963 |
| Post-treatment PSADT (days), median (range)             | 233.1 (21.9, 540.4)        | 99.4 (5279.7, 543.2)       | 0.423 |
| Change in pre- to post-treatment PSA velocity (ng ml⁻¹ per year), median (range) | –0.4 (–4.2, 0.8) | –0.2 (–2.4, 1.8) | 0.536 |

Abbreviation: PSADT, PSA doubling time.

The fact that we observed five declines and no increases ≥10% in 5mC content speaks to the possibility that disulfiram may be a weak demethylating agent; however, given potential issues with intrinsic 5mC content variability in this patient population the hypothesis of this study cannot be confirmed. This lack of robust activity coupled with the high rates of toxicity seen at the higher dose leaves us unable to recommend future study of disulfiram in this patient population. Although this study provides limited insights regarding pharmacokinetic correlations given that only 2/12 subjects had detectable plasma MeDDC levels, given that these levels were elevated in the ‘responders’, may indicate that inconsistent disulfiram pharmacokinetics may have been partly responsible for the low observed rate of demethylation.24 If developed, newer non-nucleoside DNMT inhibitors with improved pharmacokinetic properties and less toxic side effect profiles, perhaps based off disulfiram or one of its metabolite’s parent structures, should be tested in men with PCa. At this point, however, the utility of DNA demethylation as a therapeutic strategy for patients with prostate cancer remains to be proven.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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