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Final Report – DOD Concept Award - BC-024051 – J. C. Bulinski, P.I.

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Many chemotherapeutic drugs most effective in combating breast cancer work by altering microtubule (MT) dynamics, i.e., MT assembly and disassembly from tubulin protomers. The taxanes, Taxol (paclitaxel) and Taxotere (docetaxel), increase MT stability along the entire length of MTs [1]. Taxanes are widely used to halt breast tumors, because they block mitosis and metastasis, and promote apoptosis, though some breast cancers are unpredictably refractory to taxanes, even at levels high enough that toxic side-effects ensue [2]. We set out to test for MT-stabilizing drugs that act in all cell types and are mechanistically distinct from taxanes, as potentially promising new therapies. Also, if such a novel MT-stabilizing agents and Taxol were used in combination, tumor cells would be unlikely to escape or adapt to the effects of both, and additive or synergistic action of the two compounds would permit therapeutic regimens in which each was administered at low levels that precluded toxicity. Our initial working hypothesis was that histone deacetylation inhibitors could serve as novel MT-stabilizing agents that would work like Taxol, but via mechanisms distinct from Taxol.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement Of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Appended publications and/or presentations may be substituted for detailed descriptions but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Grants Officer. This approval must be obtained prior to initiating any change to the original Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research. [for figures, see following page]

- We confirmed and extended the demonstration that microtubule acetylation level affects cell motility [e.g., 3, 4]. **[see Figure 1]**
- We measured In vivo microtubule dynamics in the presence of HDAC inhibitors; using methods we had previously developed [5]; they were significantly decreased in cells whose acetylation level was increased via treatment with the broad spectrum histone deacetylase inhibitor, trichostatin A; however, microtubule stabilization was modest, equivalent to treatments with submicromolar Taxol concentrations. **[see Figure 2]** Interestingly, these results settle a conundrum raised by reports that increased microtubule acetylation increases microtubule stability [6], and two groups who had been unable to measure any stability change [3, 7].

- We also showed that increased acetylation of microtubules, but not of other cellular proteins, yielded a time-dependent increase in focal adhesion area, suggesting that heightened levels of acetylated microtubules may hinder cell migration through increasing cell adhesion, through decreasing microtubule dynamics, or through some combination of both. **[see Figure 3A, B]**
- We found that this adhesion change was reversible, and occurred both with broad-spectrum HDAC and those that are specific for the MT deacetylase; transfection to overexpress or under-express (i.e., siRNA) **[see Figure 3, C, D]**
- Our results suggest possible functions of the acetylation modification of microtubules, as well as novel mechanisms of action of histone deacetylase inhibitors.
- We have also started to cast a wider net for post-translational modifications important in tumorigenesis. To do so, we have examined *all* the post-translational modifications of α-tubulin that are known to occur **[see Figure 4]**
- We have found that, in prostate cancer cell lines, in which we made a comparison of normal, transformed, and tumor cells (both hormone-responsive and non-responsive) characteristic changes in the levels of post-translational modifications of tubulin occurred **[see Figure 5].** We suspect that this is also the case in breast cancer cells, which may have a 'signature' of altered post-translational modifications. However, our funds were depleted and the project period was over before we could test this hypothesis.

Legends to Figures:

Figure 1. Inhibition of HDAC6 decreased cell motility in non-transformed and transformed NIH-3T3 cells. (A) Motility of NIH-3T3 and **(B)** DTRas cells in a Transwell chemotactic invasion assay shows that **TSA** or **tubacin**, but not **NaB**, significantly decreased motility (significant differences from the control, i.e., $p < 0.05$, are noted by asterisks). Controls were untreated or treated with vehicle alone (**DMSO**); **Taxol** was used as a positive control. Note that, while 1 μM **Taxol** significantly inhibited motility of NIH-3T3 cells, 100 nM **Taxol** did not have a significant effect. Cell migration was quantified as the number of cells per field that successfully migrated to the other side of each gelatin-coated insert. **(C)** Micrographs show typical fields in the Transwell invasion assay.

Figure 2. HDAC6 inhibition decreases MT dynamics in vivo. (A) Typical time-lapse micrographs used to measure MT dynamics by MT end-tracking; an edge of a 3xGFP-EMTB TC-7 cell is shown in reverse contrast, with elapsed time represented as min:sec. **(B) TSA** and **Taxol** pre-treatments (30 min) decreased percentage of time that MTs were in a dynamic phase, i.e. [(total time spent polymerizing) plus (total time spent depolymerizing)] divided by (total elapsed time) x 100. **(C) TSA** and **Taxol** pre-treatments (30 min) yielded a dose-dependent decrease in MT dynamicity, i.e. [(total distance all MTs polymerize) plus (total distance all MTs depolymerize)] divided by (total elapsed time)[8]. Rates of MT polymerization **(D)** and depolymerization **(E)** were significantly decreased by treatment with **TSA**, though the decrease was less than that effected by **Taxol**. In all panels, conditions significantly different from controls ($p<0.05$ in a student's t-test) are marked with asterisks (*).

Figure 3. Altered level or activity of HDAC6 alters focal adhesion area. (A) Treatments (2 hr) of TC-7 cells with **TSA**, but not with **NaB**, increased focal adhesion area relative to control cells (**DMSO**); adhesion area was measured as percent of the cell footprint immunostained for paxillin (see Materials & Methods). **Taxol** treatment (100 nM or 1 μM) also increased adhesion area; effects were not significantly different from **TSA** treatment. Asterisks denote measurements significantly different from control ($p<0.05$). Note that cell footprint of TC-7 cells was unchanged by drug treatments (*data not shown*), as shown for NIH-3T3 cells in **Figure 2**. **(B)** Paxillin immunostaining of typical TC-7 cells, following 2 hr treatments as indicated. Bar in top left panel indicates 20 μM. **(C)** Focal adhesion area was increased in A549-HDAC6 Knock-Down cells, with decreased HDAC6 level. A549-HDAC6 Knock-Down cells showed greater adhesion area than A549-control cells under control conditions (**DMSO**) and in the presence of **TSA** (*** shows significant differences), but not in the presence of **Taxol**. Similarly, A549-HDAC6 Knock-Down cells were significantly increased in adhesion area by **TSA** (**), but not by **Taxol** treatment; control A549 cells showed adhesion area significantly increased (*) in response to either **TSA** or **Taxol**, compared to the control (**DMSO**). **(D)** Focal adhesion area was decreased in NIH-3T3-HDAC6 cells. Expression of transfected HDAC6 significantly decreased focal adhesion area compared to control NIH-3T3 cells; both cell lines responded significantly to both **TSA** and **Taxol** (asterisks denoting significance are as described in **(C)**.

Figure 4. Schematic illustrating the other multiple posttranslational modifications of α-tubulin. All post-translational modifications, with the exception of palmitoylation were examined in breast cancer cells.

Figure 5. Distinct profiles of α-tubulin posttranslational modifications are observed in normal human prostate epithelial cells and prostate cancer cells. The cell lines examined were normal human prostate epithelial cells (PrEC), immortalized normal human prostate epithelial cells (PZ-HPV-7), androgen-dependent prostate cancer cells (LNCaP) and androgen-independent prostate cancer cells (PC3). Cells were harvested prior to confluence, proteins were electrophoresed and western blotted with antibodies specific for the various posttranslational modifications illustrated in Fig. 4.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- Tran, A., Hubbert, C., Kawaguchi, Y., Yao, T-P, and Bulinski, J. C. Increased acetylation of microtubules increases cell adhesion and inhibits cell migration. Manuscript submitted, 2005.
- Tran, A. and Bulinski, J. C. Increased acetylation of microtubules increases cell adhesion and inhibits cell migration. American Society for Cell Biology, Abstract to be presented 7 December, 2004.
- Tran, A. Recinos, D. and Bulinski, J. C. Increased acetylation of microtubules increases inhibits cell migration, Abstract to be submitted for 2005, Era of Hope Meeting., 10-12 June, 2005.

CONCLUSIONS: Summarize the results to include the Importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

- We conclude that acetylation-induced microtubule stabilization makes a significant contribution to decreasing cell motility.
- We strongly suggest that microtubule effects of HDAC inhibitors are a vital part of their anti-tumor action, and these effects and mechanisms *must* be studied further
- We conclude HDAC inhibitors used clinically are promising compounds for increasing cell adhesionof tumor cells, and therefore working as potential anti-metastatic agents.
- We demonstrate that the study of HDAC inhibitors is of vital interest to basic researchers and clinicians, and that there is moe work to be done in this emerging area.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Not applicable. The manuscript cited above is just submitted, and the abstract is *in press.*

Personnel: Include a list a list of personnel (not salaries) receiving pay from the research effort.

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Figure 1: BC024051 Final Progress Report

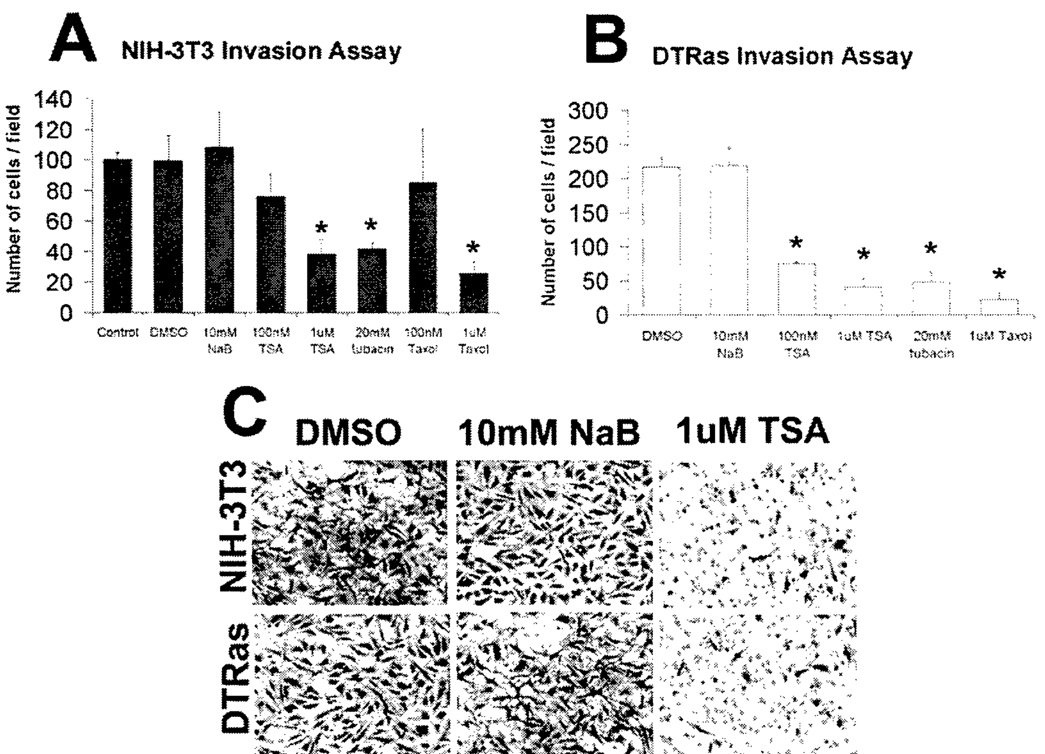

Figure 2: BC024051 Final Progress Report

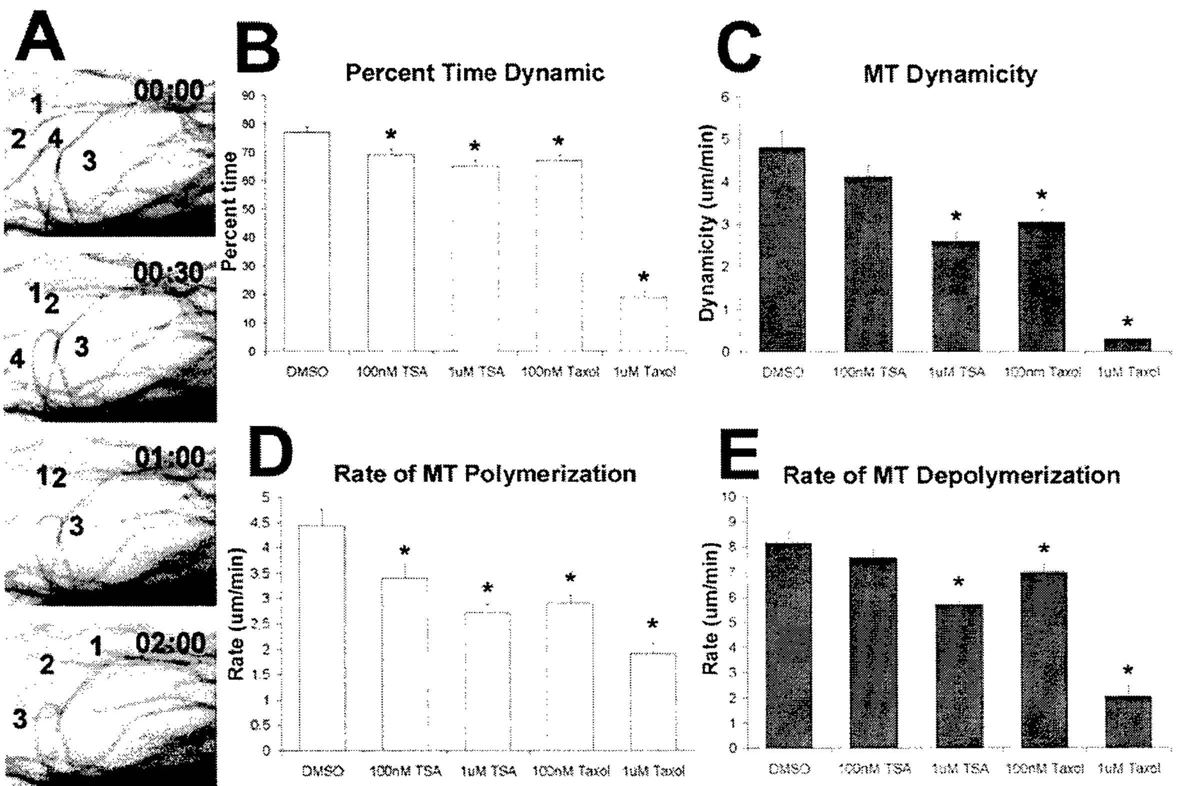

Figure 3: BC024051 Final Progress Report

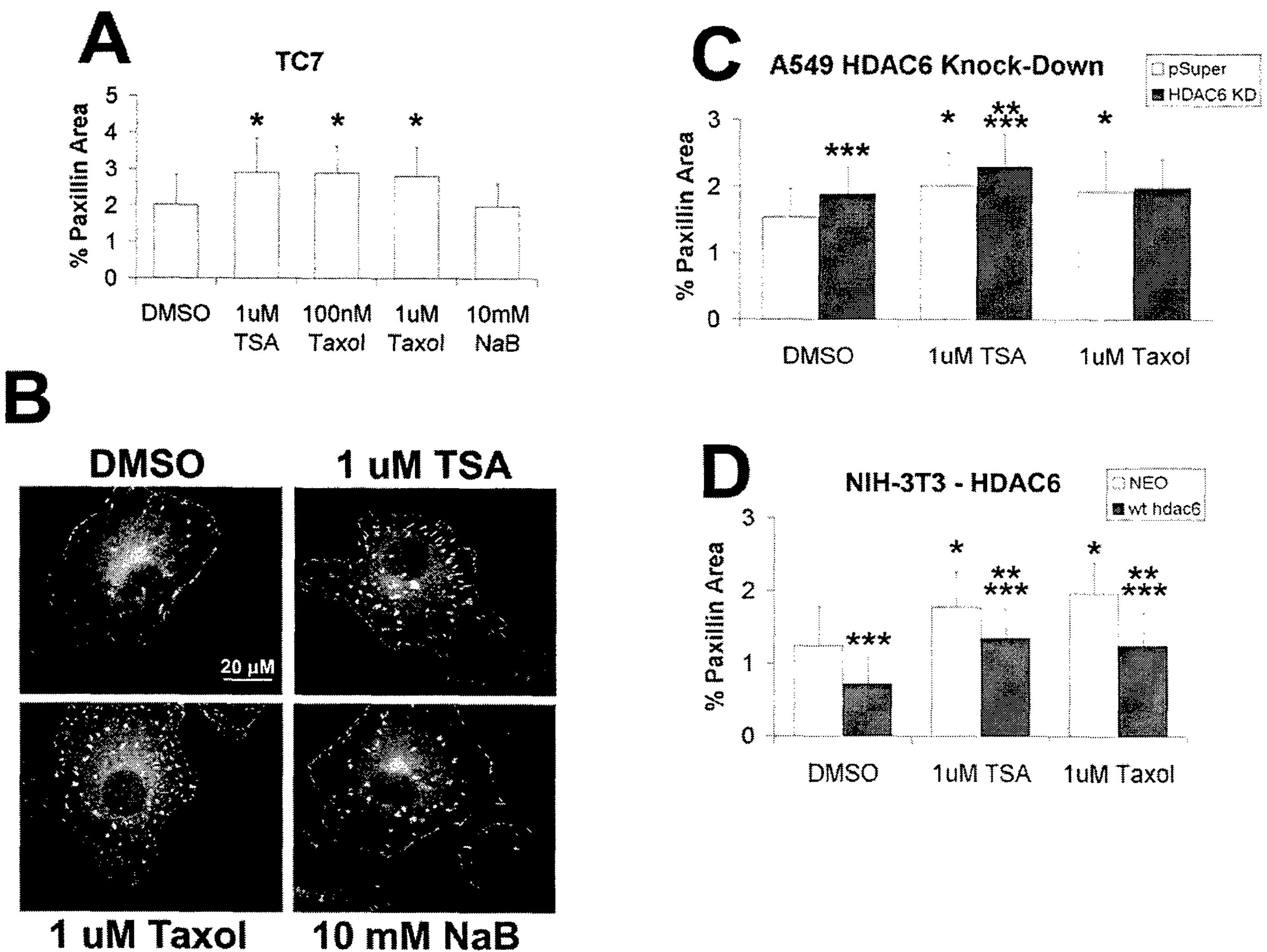

Figure 4: BC024051 Final Progress Report

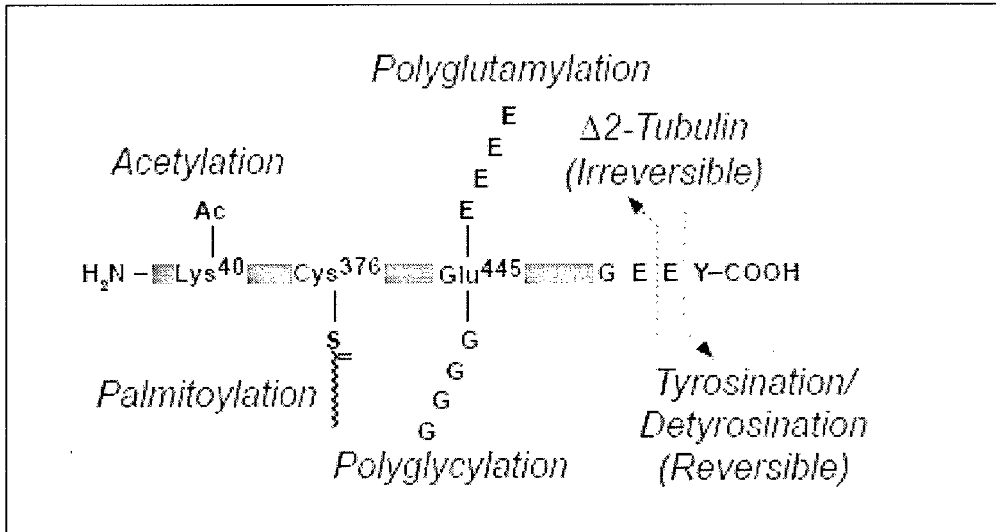

Figure 5: BC024051 Final Progress Report