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14. ABSTRACT
This predoctoral fellowship supported research on apoptotic signaling in response to key breast cancer chemotherapeutics, microtubule inhibitor (MTI). The funding also supported educational activities that trained the Principal Investigator (PI) as a physician-scientist. MTIs are the most actively used agents for metastatic and adjuvant breast cancer therapy, yet their use is limited by resistance and side effects. MTIs activate a kinase that phosphorylates and inactivates Bcl-xL, an anti-apoptotic protein that can cause resistance to chemotherapeutic agents. Overall, our data show that Cdk1/cyclin B1 phosphorylates Bcl-xL *in vitro* and cell culture models following MTI treatment, during normal mitosis, and during prolonged mitosis without MTI treatment. Published data also indicate that Bcl-xL phosphorylation increases tumor cell apoptosis. Presented here are the final data on the clinical research study of Bcl-xL and Bcl-2 phosphorylation pre- and post-taxane treatment in patients who have breast cancer. Remaining research and educational activities are discussed.

15. SUBJECT TERMS

Microtubule inhibitors, breast cancer, Bcl-xL, vinblastine, Cdk1, cyclin B, mitotic arrest

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INTRODUCTION: This training grant set out to identify and characterize the Bcl-xL kinase as a potential mediator of breast tumor and normal cell sensitivity to actively used chemotherapeutics, microtubule inhibitors (MTIs) and, to train the Principal Investigator (PI) as a future breast cancer physician-scientist. MTIs are the most actively used agents for metastatic and adjuvant BC therapy, yet their use is limited by resistance and side effects (1-2). Bcl-xL is an anti-apoptotic protein that causes resistance to chemotherapeutic agents when overexpressed in many tumor types. Following MTI treatment, Bcl-xL is phosphorylated and inactivated (3-4), representing a possible molecular pathway that leads to chemotherapy sensitivity via Bcl-xL inactivation. The kinase responsible for this phosphorylation was unknown at the initiation of this project. The specific aims were (1) develop and optimize a robust and specific assay for Bcl-xL kinase activity and (2) purify and identify the Bcl-xL kinase. The data for the successful completion of the aims was presented in the 2009 annual report. It presented the development of a Bcl-xL kinase assay and preliminary *in vitro* identification as Cdk1/cyclin B1, were reported in 2009. The 2010 annual report presented confirmation that Cdk1/cyclin B1 phosphorylated Bcl-xL in cultured KB-3 cells and partially sensitized them to apoptosis stimuli. In addition, a

pilot clinical study was presented. The project analyzes Bcl-2 post-translational modifications in peripheral blood mononuclear cells (PBMCs) from patients who have breast cancer (BC) before and after taxane therapy, where PBMCs would serve as a surrogate marker for breast tumor sensitivity or predictor of bone marrow suppression (i.e. apoptosis of hematopoietic precursors). The final data on this project is presented here. The budget period has ended and thus this final report is much shorter and presents the final round of data.

The specific breast cancer training plan was designed to educate the PI as follows. 1) Understand the molecular biology behind tumor cell death following treatment with anti-cancer agents; 2) Translate that work into clinical studies; 3) Learn the clinical side of breast cancer, through clinical and inpatient medical school education; and 4) Learn collaboration and communication skills for work with both clinical and basic science breast cancer researchers through a monthly Breast Cancer Focus Group (designed by the PI), basic research, research seminars, dissertation writing, publication, and presentations at professional meetings and departmental seminars.

BODY:
Currently, I have begun my residency in Pathology at Columbia University, New York Presbyterian Hospital. This report summarize the final round of data and education activities

The last annual update reported that Cdk1/cyclin B1 was the MTI-activated Bcl-xL kinase in the KB-3 cell culture model. Three specific, validated Cdk inhibitors (6-7) were used in cells exquisitely synchronized in mitosis and treated with a MTI, vinblastine, so as to not block progression to mitosis and to avoid inhibition of non-mitotic Cdk's (i.e. Cdk 2, 4, 6). This validated preliminary *in vitro* data from the FL62 peptide kinase assay. Subcellular localization studies also showed that Cdk1/cyclin B was present in purified mitochondria and phosphorylated FL62 only from MTI-treated KB-3 cells. Immunofluorescence confirmed mitochondrial localization of Cdk1 and cyclin B following vinblastine treatment.

Since transient and partial Bcl-xL phosphorylation was a normal mitotic event, we analyzed phosphorylation in synchronized cells when cyclin B degradation was inhibited by the proteasome inhibitor MG132. Indeed, this blocked cyclin B degradation, maintained Cdk1/cyclin B activity, and enhanced Bcl-xL phosphorylation to levels comparable to MTI treatment. Using this logic we subsequently studied the effect of overexpressing non-degradable cyclin B. Those results are presented again below. All of these data, from aim 1 through the apoptosis studies, comprised our publication in the Molecular and Cellular Biology Journal, available on PubMed and the Molecular and Cellular Biology Journal (see reference 5). The publication was also attached to the 2010 annual report.

In this report, we present two final pieces of data: (1) apoptosis data that was discussed in 2010 but not presented clearly and (2) final data on the pilot clinical study of Bcl-2 protein phosphorylation in PBMCs from patients who have metastatic BC before and after taxane therapy.

Other information presented here includes work obtaining IRB-approval for a side project studying the understanding of tissue banking among African-American women who have BC. This was work funded through a UAMS scholarship, but initiated through breast cancer focus group.

Data that support the text here are also in the "APPENDICES", specifically "APPENDIX 1." There are also publications and posters listed which include data from my project and side projects related to my thesis work.

Basic research/laboratory data
We analyzed the role of Bcl-xL phosphorylation in Cdk1/cyclin B1 pro-apoptotic signaling, a controversial issue regarding Cdk1 function (8-10). KB-3 cells were transfected with wild-type or non-degradable cyclin B1 (cyclin B1 (R42A)-GFP) to show that prolonged Cdk1 activity leads to apoptosis. Non-degradable cyclin B1 induced significantly more apoptosis than GFP transfectants. Wild-type cyclin B1 did as well, but significantly less (Fig. 1A). Next KB-3 cells were co-transfected with non-degradable cyclin B1 and either wild-type, phospho-defective (S62A), or a phospho-mimic (S62D) mutant of Bcl-xL. Transfection with S62A Bcl-xL significantly blocked apoptosis induced by non-degradable cyclin B1 ($P \leq 0.001$ compared to wild-type Bcl-xL), while S62D did not ($P \leq 0.442$ compared to wild-type Bcl-xL) (Fig. 1B). Apoptosis protection was also not due to different expression levels (data not shown).

Translational research study

We continued the clinical protocol entitled "Alterations in the Bcl-2 Family Proteins in the Peripheral Blood Following Treatment with Taxanes in Patients with Breast Cancer." The study was approved by the UAMS Institutional Review Board (IRB) in 2009. The goal is to determine if Bcl-xL, Bcl-2, and Mcl-1 are phosphorylated in peripheral blood mononuclear cells following taxane treatment. Then we will analyze if that phosphorylation correlates with a disease response or non-response, or with the side effect of bone marrow suppression. Peripheral blood mononuclear cells may act as a surrogate marker for breast tumor sensitivity or side effects if the hypothesis is affirmed. If it is not – i.e. no Bcl-2 protein phosphorylation – then it can serve as a negative internal control when studying actual tumor samples.

The results from three patients who have breast cancer did not show Bcl-xL or Bcl-2 phosphorylation before or after taxane therapy in PBMCs (Fig. 2 and Table 1). While Bcl-xL levels were absent in one sample after taxane therapy (Fig. 2), it is unclear if it is due to a change in cell population. In addition, cyclin B1 levels did not increase (Fig. 2), suggesting that the cells were not mitotically arrested, presumably because most, if not all, cells are fully differentiated and no longer dividing. The data are collated in Table 1 here.

Table 1. *Phosphorylation of Bcl-2 proteins in peripheral blood mononuclear cells in patients who have metastatic breast cancer before and 16-24 hours following taxane therapy. (n = 3). Unphosphorylated (UP); phosphorylated (P).*

Patient #	Bcl-xL phosphorylation Status	
	Pre-taxane	16-24 h post-taxane
1	UPi	UPi
2	UPi	UPi
3	UPi	UPi

We have decided, at this point, to end the study because the data do not justify the efforts of the patients (i.e. return to the hospital 1-day post-treatment) and other more direct studies (i.e. breast tumor tissue or bone marrow) will be more justified. Cell sorting and flow cytometry techniques, for example, could be employed to determine if mitotic cells are present and, if so, isolate them for analysis of Bcl-xL, Bcl-2, and Mcl-1phosphorylation. However, the cell numbers will likely be low and probably low yield. Alternatively, to study the breast tumor tissue, biopsy of the tumor cell will be necessary both pre-and post treatment in the setting of neo-adjuvant therapy, which would come before surgical excision. Alternatively, one could study bone marrow hematopoiesis to predict the side effects of bone marrow suppression (BMS). These cells would be advantageous because they are proliferating, or cycling. They are thus more likely to undergo mitotic arrest, which our studies show is required for Bcl-2 protein phosphorylation. Those patients with Bcl-2 protein phosphorylation, and even Cdk1/cyclin B activity, may be more susceptible to BMS. A patient with greater Bcl-2 protein phosphorylation in hematopoietic cells may be more likely to have fever, neutropenia than others, for example. While effective treatment will supersede BMS, better predicting who will have this side effect can prepare a clinician for post-treatment management decisions.

This clinical data and the basic research data above were presented as a poster at the American Society for Clinical Investigation/American Physician Scientists Association 2010 Annual Meeting.

Specific training tasks

My training proceeded as planned in the original proposal. Scientifically, the final version of our paper in the Molecular and Cellular Biology Journal was published, and the pilot clinical study was completed. In addition, Dr. Chambers and I were invited to write a review for the journal Cell Division discussing mitosis, mitotic arrest, and apoptosis. The manuscript is currently in preparation. I continue to discuss with Dr. Chambers new data obtained by members in his laboratory. For career development, I presented a poster at the American Society for Clinical Investigation/American Physician Scientists Association and the Central Society for Clinical Research annual meetings in April 2010, continued medical school rotations (several within the oncology), and continued to organizing the UAMS Breast Cancer Focus Group.

The study is entitled "Understanding of Bio-Banking in African American Patients who have Cancer Understanding of Bio-Banking in African American Patients who have Cancer," was continued. As a reminder, the study asks if African American (AA) patients who have breast cancer understand bio-banking and if that influences their decision to donate. Currently, AAs donate their tissues for research at lower rates than Caucasian (11) populations and they also express less trust of physicians (12). Patients will be asked about their understanding of tissue donation for research, and then educated about it afterwards. Differences

between racial populations will be compared. The study will be part of the Biospecimen Management Program (**BMaP**) study at UAMS. The **BMaP** study is funded by the Cancer Research Network at the NCI directed at UAMS by breast surgical oncologist Ronda Henry-Tillman, M.D. **BMaP** is a multi-center study that aims improve the quality and quantity of biological specimens collected from AA and minority populations who have cancer in the mid-south region. The study came about when applying for the Bruce and Brandon Lee scholarship in the UAMS Medical Humanities department. The scholarship is dedicated to better understanding ethical and social issues involved of oncology. Students can propose any idea they wish, and only one is offered a scholarship. To find a proposal, I sought out Dr. Henry-Tillman after one of her administrators presented their work at a Breast Cancer Focus Group meeting. There, she suggested this idea, which I then independently researched. I was fortunate to be offered that one scholarship. IRB approval was obtained this spring. I am coordinating with Dr. Henry-Tillman and her team to determine how the study will be conducted in my absence.

Finally, the Division of Hematology and Oncology breast cancer focus group continues to meet monthly. I transferred coordinating information to the division administrator and to Dr. Thomas Kelly, an advisor who was on my dissertation committee and a breast cancer researcher. Dr. Laura F. Hutchins, my previous clinical mentor, continues to oversee these activities as the Chair of the Division of Hematology and Oncology.

KEY RESEARCH ACCOMPLISHMENTS:
Basic Research
- Phosphodefective Bcl-xL blocks cell death induced by non-degradable cyclin B1.
- Preparation of a review of discussing mitosis, mitotic arrest, and apoptosis for the journal Cell Division

Clinical Research
- Transferred responsibility of the coordinating the monthly Breast Cancer Focus Group (BCFG) at UAMS.
- Co-author of the clinical research protocol "Alterations in the Bcl-2 Family Proteins in the Peripheral Blood Following Treatment with Taxanes in Patients with Breast Cancer." Accepted by the UAMS Institutional Review Board **(Study protocol #109857).**
- Bruce and Brandon Lee Scholarship, UAMS Department of Humanities. Advisors: Chris Hackler, PhD and Rhonda Henry-Tillman, M.D.
  - Title: Understanding of Bio-Banking in African American Patients who have Cancer (IRB approval obtained).
  - Coordinating logistics of conducting this study.

REPORTABLE OUTCOMES:
Publications, Posters, and Presentations
- **Terrano, D.T**. and Chambers T.C. Cyclin-Dependent Kinase 1-Mediated Bcl-xL/Bcl-2 Phosphorylation Acts as a Functional Link Coupling Mitotic Arrest and Apoptosis. Mol. Cell. Biol., 2010, 30(3), 640-656. PMID 19917720.
- **Terrano, D.T**. and Chambers T.C. Mitosis, Mitotic arrest, and Apoptosis in response to microtubule inhibitors, Cell Division, *Invited review* (Manuscript in preparation).
- Rong Chu, **David T. Terrano** and Timothy C. Chambers. Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis by phosphorylation of Mcl-1, promoting its degradation and freeing Bak from sequestration, Manuscript submitted to Biochemical Pharmacology.
- **D Terrano**, JJ Joheim, N Sakurikar, I Makhoul, LF Hutchins and TC Chambers. CDK1/Cyclin B1 Mediate Bcl-xL/Bcl-2 Phosphorylation is a Pro-Apoptotic Signal Following Mitotic Arrest and Microtubule Inhibitor Treatment. Abstract Number 79. The American Society of Clinical Investigation/American Association of Physician Scientists Joint Meeting, 2010, Chicago, IL.
- **D Terrano**, JJ Joheim, N Sakurikar, I Makhoul, LF Hutchins and TC Chambers. CDK1/Cyclin B1 Mediate Bcl-xL/Bcl-2 Phosphorylation is a Pro-Apoptotic Signal Following Mitotic Arrest and Microtubule Inhibitor Treatment. Poster 74. Central Society for Clinical Research and Midwestern Section American Federation for Medical Research Combined Annual Meeting, 2010, Chicago, IL.

CONCLUSION: The data from this proposal accomplished specific aims 1 and 2. In aim 1, we developed an *in vitro* Bcl-xL kinase with a novel peptide substrate FL62. Cdk1/cyclin B phosphorylates purified from MTI-treated cells extensively phosphorylates FL62. In aim 2, we confirmed in cells that Cdk1/cyclin B phosphorylates Bcl-xL and Bcl-2, and, in recent data (not shown) Mcl-1. Subsequent studies confirmed that Cdk1/cyclin B phosphorylates these proteins normally during mitosis, although transiently and partially. Together, these data suggest that Cdk1/cyclin B1 phosphorylation of these proteins may act as a sensor for the duration of mitosis where prolonged mitosis leads to maximal phosphorylation and inactivation of Bcl-xL, Bcl-2, and Mcl-1. In turn, cells are more sensitive to cell death signals perhaps by the pro-apoptotic Bcl-2 proteins which Bcl-xL, Bcl-2, and Mcl-1 directly inhibit. The cells then begin apoptotic signaling, exit mitosis, and die. While past and current data indicate Cdk1/cyclin B1 protects against mitosis by phosphorylation and inhibition of caspase-9 (9) and phosphorylation and stabilization of pro-survival protein survivin (10), the duration of mitosis was critical to this function in both studies. Experiments are underway to understand this and the link between Cdk1/cyclin B1 and apoptosis.

Interestingly, new experiments from Dr. Chambers' lab are studying Bcl-xL phosphorylation in cells after prolonged activation of the spindle assembly checkpoint through inactivation of Cdc20. Cdc20 inhibits the spindle assembly checkpoint when cells are prepared for anaphase. Inactivating it through siRNA knockout, prevents this inhibition, and the spindle assembly checkpoint remains active. There is published data that Mcl-1 phosphorylated by Cdk1 is degraded through a Cdc20-dependent pathway (11) These studies are critical for determining the role of Cdk1/cyclin B, the spindle assembly checkpoint, and Bcl-2 protein phosphorylation during mitotic arrest as a controlled model of MTI treatment.

Finally, the clinical studies indicate that peripheral blood mononuclear cells are not surrogate markers for Bcl-2 protein phosphorylation, which was undetectable in these cells.

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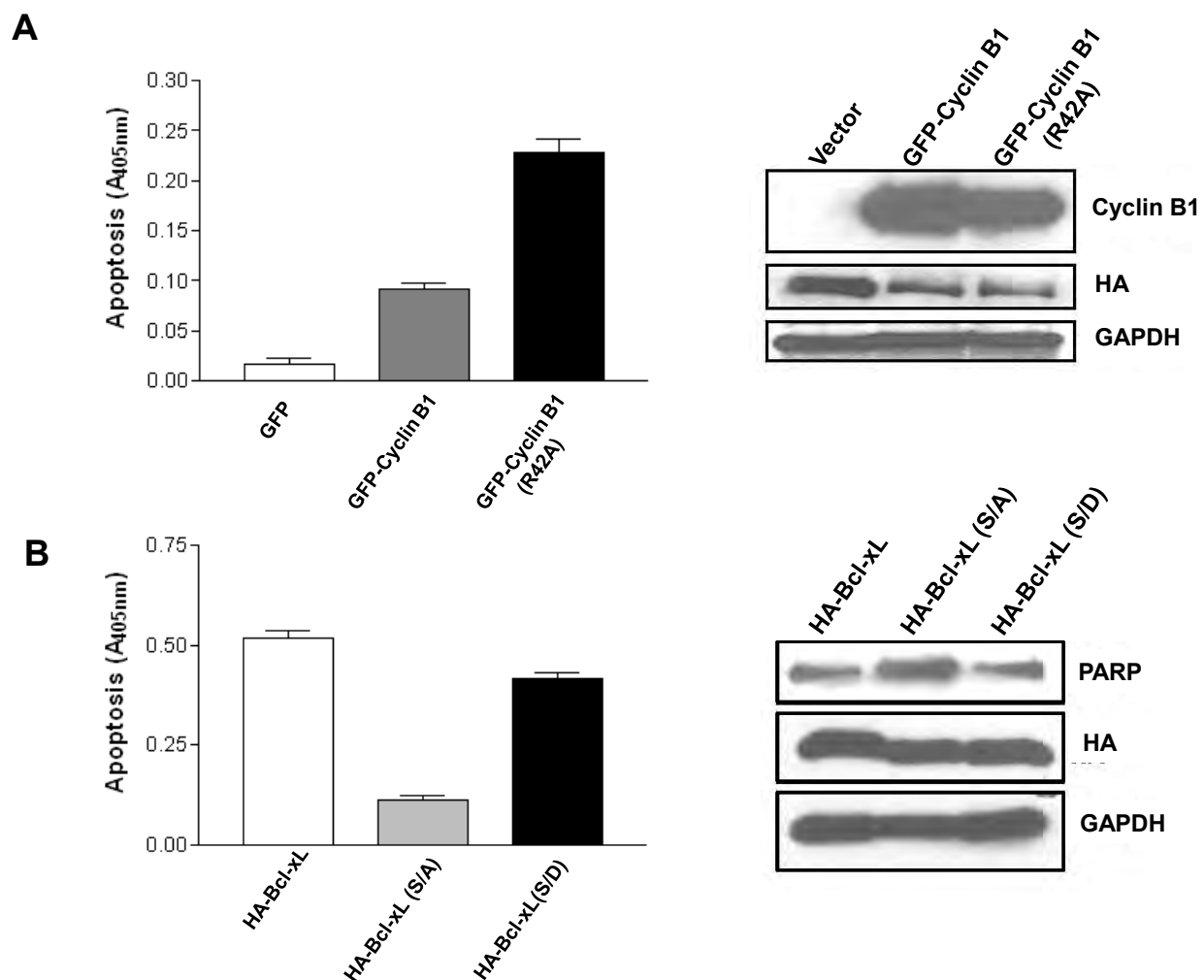
A

B

Figure 1.   Phospho-defective Bcl-xL blocks cell death induced by non-degradable cyclin B1. A. KB-3 cells were transfected with 1 µg plasmid DNA encoding GFP vector, wild-type cyclin B1-GFP, or cyclin B1(R42A)-GFP. *Left,* 48 h post-transfection, cells were subjected to apoptotic cell death ELISA assay (mean ± S.D., n = 6). *Right,* extracts were made and subjected to immunoblotting for cyclin B1 (24 h post-transfection), PARP (48 h post-transfection) or GAPDH. **B.** KB-3 cells were transfected with 1 µg plasmid DNA encoding cyclin B1(R42A)-GFP together with 1 µg plasmid DNA encoding either wild-type (wt), phospho-defective (S62A) or phospho-mimic (S62D) HA-Bcl-xL. *Left,* 48 h post-transfection, cells were subjected to apoptotic cell death ELISA assay (mean ± S.D., n = 6). * p $\leq$ 0.001 versus wild-type Bcl-xL; ** p $\leq$ 0.422 versus wild-type Bcl-xL. *Right,* extracts were made and subjected to immunoblotting for PARP (48 h post-transfection), HA-tag (48 h post-transfection) or GAPDH.
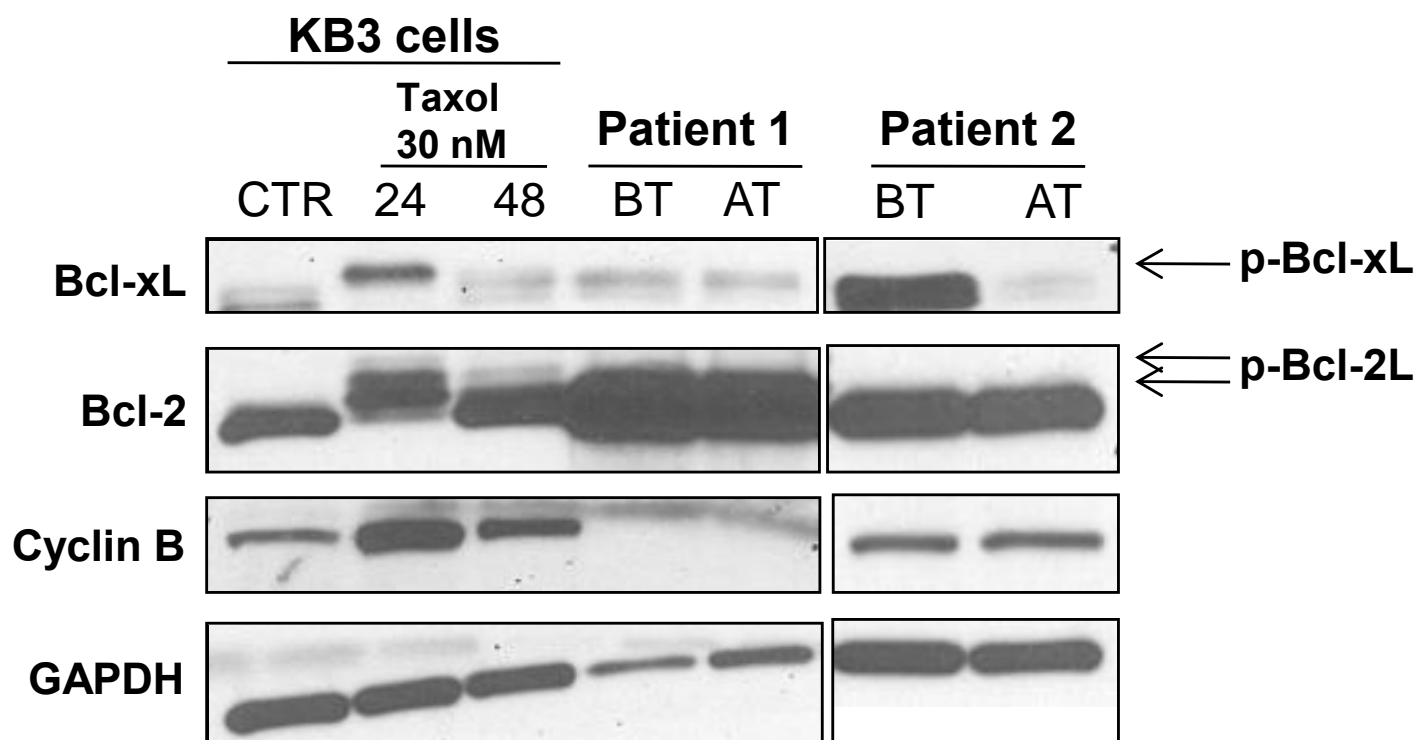
Figure 2. Analysis of Bcl-2 protein phosphorylation in peripheral blood mononuclear cells in patients with breast cancer treated with a taxane. Patients who have metastatic breast cancer and scheduled for taxane treatment were recruited. UAMS IRB approval was obtained and each patient signed an informed consent. Peripheral blood was collected immediately before treatment and 16-24 hours after. The perierpheral blood mononuclear cells were isolated using a Ficoll gradient. Protein concentration was quantified and similar amounts applied to SDS-PAGE and immunoblotted for the proteins indicated on the left. Samples from before taxane (BT) and after taxane (AT) were analyzyed side by side. Extracts from KB-3 cells left untreated (CTR) and those treated with 30 nM Taxol for 24 and 48 hours were analyzed as controls for unphosphorylated (CTR and 48h) and phosphorylated (24 h) Bcl-xL and Bcl-2. Arrows indicated the slower migrating, and hence phosphorylated, Bcl-xL and Bcl-2. GAPDH is a loading control. Two representative patient samples are shown (n = 3).