Abstract # 4837

Pro-apoptotic Activity of the Chinese Medicinal Herb *Scutellaria barbata* (BZL101) on Breast Cancer Cells

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Background

- Annually, over 200,000 women in the US are diagnosed with breast cancer, the 2nd leading cause of cancer deaths for women.
- Despite advances in breast cancer treatments, current regimens lead to toxic side effects and are mostly ineffective against metastatic breast cancer. Therefore, therapies specifically targeting cancer cells, while leaving normal cells intact and functioning, are urgently needed.
- Botanical medicines are frequently sought and used by cancer patients, yet few studies have evaluated their safety and efficacy; as well as their mechanisms of action.
- Bionovo Inc. has completed a phase I clinical trial for *Scutellaria barbata* (BZL101) in a heavily pretreated patient population with metastatic breast cancer and showed that it was safe and had a favorable toxicity profile. In addition, BZL101 demostrated encouraging clinical activity (Rugo *et al.,* 2006). Currently, a Phase 1/2 trial is underway to assess safety, feasibility, optimal dosing and preliminary efficacy of BZL101 for the treatment of metastatic breast cancer.
- Preclinical evidence thus far has shown that BZL101 exerts selective, anti-tumor, pro-apoptotic/necrotic activity against breast cancer cells.
  - BZL101 has selective anti-proliferative activity against a panel of cancer cell lines derived from various origins while showing minimal effects on normal human mammary epithelial cells or normal human fibroblasts (Campbell *et al.*, 2002, Shoemaker *et al.,* 2005).
  - In addition, oral-intake of BZL101 has been shown to prevent tumor formation in a mouse mammary carcinoma xenograph model (unpublished data).
  - Treatment of breast cancer cells with BZL101 induces both necrotic and apoptotic death and it is accompanied by a G2/M cell cycle arrest (Rugo *et al.*, 2006).
  - BZL101 induces rapid and strong depolarization of mitochondrial transmembrane potential (MTP). In addition, exposure of breast cancer cells to BZL101 rapidly increases the production of reactive oxygen species (ROS) (Rugo *et al.,* 2006).
- Here, we present data on the potential mechanism of action for the death-inducing effect of BZL101 on breast cancer cells.

References

1. Campbell *et al.* (2002) *Anticancer research,* **22,** 3843-3853.
2. Rugo *et al.* (2006) *Breast Cancer Res Treat*, First published ahead of print as DOI 10.1007/s10549-006-9430-6.
3. Shoemaker *et al.* (2005) *Phytother. Res.* **19**, 649-651.

Methods

BZL101 preparation BZL101 is an aqueous extract of the aerial part of *Scutellaria barbata* D. Don of the Lamiaceae family (Campbell *et al.*, 2002). BZL101 was used at 1:200 dilution for 20-24 hours in all assays unless indicated otherwise. **Mitochondrial transmembrane potential (MTP)** was measured by fluorescence emitted by aggregates of JC-1(Invitrogen) dye in mitochondria. **DNA fragmentation analysis** was done by staining ethanol-fixed cells with propidium iodide and DNA content analyzed using flow cytometry. Analysis of apoptotic versus necrotic death was done by staining cells with Annexin V-Alexa Flour 488 (apoptotic) and propidium idodide (necrotic), followed by flow cytometry analysis. **Assessment of glycolysis** was done using two assays: (1) measurement of *de novo* synthesis of intracellular lactate using magnetic resonance spectroscopy (MRS). Briefly, cells were labeled with 5 mM [1-^{13}C] glucose (last 5h) and subsequently extracted using 12% PCA. Proton-MRS experiments of cell extracts and media were performed using a Varian INOVA 600MHz spectrometer equipped with 5-mm HCN-PFG probe. A standard water presaturation pulse program was used; spectra were obtained at 12ppm spectral width, 32K data arrays, 64 scans with 90-degree pulses applied every 15 sec. Trimethylsilyl propionic-2,2,3,3,-d4 acid was set at 0 ppm for chemical shift reference and was used as an external standard for metabolite quantification. All data were processed using the TOPSPIN and WINNMR programs (Bruker). (2) measurement of lactate dehydrogenase activity using DHL cell viability and proliferation kit (AnaSpec) after 2 hours of BZL101 treatment. **Microarray analysis** was done using RNA isolated from SKBR3 and BT474 treated with BZL101 for 4 hours. Total RNA was sent to Phalanx Biotech Group and reverse-transcribed cDNA was hybridized to Human OneArry (Phalanx Biotech). Data were normalized using quantile normalization method. Genes up and downregulated >=1.7 fold were clustered using Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools. **Comet assay** was done using CometAssay kit (Trevigen) following manufacture's instruction.

Results

Table 1. Sensitivity of cell lines to BZL101 correlates with their mitochondrial transmembrane potential (MTP)

Breast cancer cells — Normal fibroblasts

Cell Line[a]	MDA-MB 468	SKBR3	BT474	IMR90
% Cell Death[b]	50	44	15	5
MTP ($\Delta\Psi$M)[c]	951	769	156	289

[a] MDA-MB468, SKBR3 and BT474 are human breast cancer cell lines. IMR90 is a human normal fibroblast line.
[b] Cells were treated with BZL101 at 1:150 dilution for 3 days, after which cell death (DNA fragmentation) was measured for DNA content stained with PI and analyzed by flow cytometry.
[c] MTP was determined using JC-1 dye in live cells and analyzed using flow cytomery. Relative arbitrary units for MTP were indicated for each cell line. Tumor cells that rely heavily on glycolytic energy generally have higher MTP.

Results

Figure1. BZL101 inhibits glycolysis

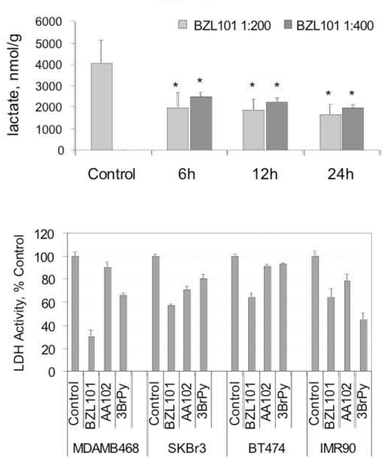


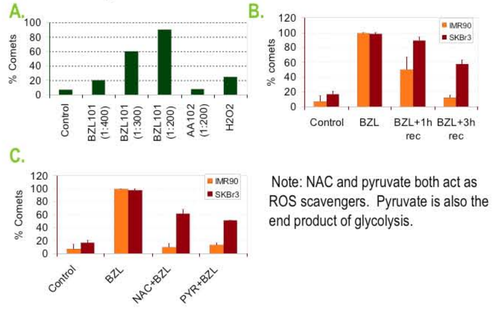
Figure 1. BZL101 inhibits glycolysis. A. Intracellular ^{13}C-lactate concentration in BZL101 treated SKBr3 cells. Cells were labeled with [1-^{13}C]glucose and acid-precipitated cell extracts were measured for *de novo* lactate synthesis using magnetic resonance spectroscopy. The values are given as means± SD of three experiments. *p<0.05 vs. untreated control. B. Lactate dehydrogenase (LDH) activity in cells treated with various herbs (at 1:200 dilution) for 2 hours. AA102 is another herbal extract developed by Bionovo Inc., and 3-Bromo-pyruvate (3BrPy) is a glycolysis inhibitor (used at 150 μM).

Table 2. Coordinated changes in gene expression as a result of BZL101 treatment on breast cancer cells

	Up-regulated clusters	Down-regulated clusters
SKBR3 only	93 genes Apoptosis NFkB pathway Cell cycle arrest - DNA damage response - Cell cycle checkpoint	87 genes Cell cycle (division) Cytoskeleton organization Cell mobility
BT474 only	47 genes Amino acid transport Nucleoside-triphosphatase activity	0 gene
SKBR3 and BT474	13 genes Xenobiotic response Oxidative response	27 genes Cell cycle control

Results

Figure 2. BZL101 induces DNA damage that is rescued by ROS scavengers



Note: NAC and pyruvate both act as ROS scavengers. Pyruvate is also the end product of glycolysis.

Figure 2. A. BZL101 induces DNA damage in a dose-dependent manner. DNA damage was assessed using Comet assay. B. Normal fibroblasts repair BZL-induced DNA damage faster than BZL-sensitive breast cancer cells. Cells were treated with BZL101 for 15 minutes and allowed to recover by replacing BZL101 containing media with fresh media. Cells were harvested for Comet assay at 1 hr and 3 hr after BZL101 removal. C. ROS scavengers rescue DNA damage in BZL treated cells. Cells were pre-treated with 10mM N-acetyl cysteine (NAC) and 10 mM pyruvate (PYR) for 30minutes, followed by BZL101 treatment for 15 minutes before cells were harvested for Comet assay.

Figure 3. BZL101 induces rapid activation of PARP (a DNA repair protein), resulting in necrotic cell death

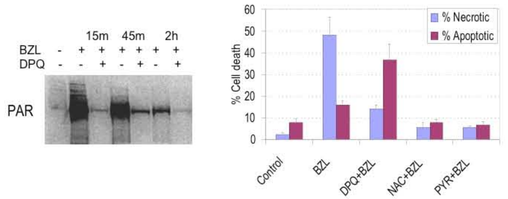


Figure 3. BZL101 induces rapid Poly(ADP-ribose) polymerase (PARP) activation which results in depletion of intracellular NAD and ATP, as well as subsequent necrotic death in breast cancer cells highly dependent on glycolytic energy. A. Western blot analysis of poly(ADP-ribose) (PAR), end-product of PARP enzymatic activity, on SKBR3 cells treated with BZL101 with or without 25 μM DPQ, a PARP inhibitor. B. PARP inhibition with DPQ results in shifting mode of death from necrosis to apoptosis. NAC and Pyruvate (PYR) reduce DNA damage caused by BZL101 and rescue cells from both necrotic and apoptotic death.

Results

Figure 4. Activity-guided isolation studies are on-going to identify the active compound(s) in BZL101

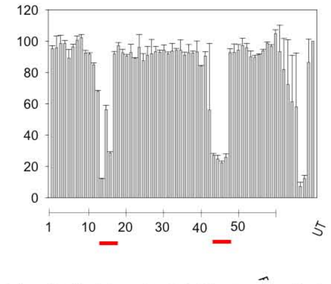


Figure 4. Activity-guided isolation using BZL101 extract identified active fractions inhibiting breast cancer cell growth. These fractions were obtained by passing aqueous (methanol/H_2O) extract of *Scutellaria barbata* through a HP20 column. Fractions were collected and analyzed using Cyquant assay to measure growth inhibition. Active fractions (▬) will be pooled and further fractionated.

Summary

- BZL101 treatment selectively targets breast cancer cells with higher mitochondrial transmembrane potential (MTP); these cells presumably rely more on glycolysis for energy (Warburg effect). BZL101 inhibits glycolysis as early as 2 hours of treatment. The most significant inhibition of lactate dehydrogenase activity was observed in cells with highest MTP.
- The effects of BZL101 on cell death/glycolysis result at least in part from coordinated changes in the expression of genes involving in oxidative/DNA damage, apoptosis and cell cycle control.
- BZL101 induced ROS production presumably causes oxidative DNA damage.
  - Normal fibroblasts repair BZL101-induced DNA damage faster than BZL101-sensitive breast cancer cells, potentially responsible for the selectivity of BZL101.
  - DNA damage induces rapid activation of poly(ADP-ribose) polymerase (PARP) that depletes intracellular NAD and ATP, and subsequently results in necrotic death. Inhibition of PARP shifts the mode of death induced by BZL101 from necrosis to apoptosis.
- Activity-guided isolation studies are on-going to identify the active compound(s) mediating the anti-tumor effects of BZL101.