Abstract
#3359

Progress on Development of a Multiplex Panel of 15 Biomarkers to Support the Development of Anticancer Drugs Targeting Apoptosis

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Introduction

Regulators of apoptotic cell death are high-interest targets for cancer therapy, and several mechanism-based investigational agents in clinical development directly target the molecular components of apoptosis pathways. We describe development and validation of a multiplex panel of biomarkers for quantifying the commitment, onset, and induction of apoptosis by the intrinsic pathway (**Figure1**). Quantitation of these pharmacodynamic biomarkers in early clinical trials will assist hypothesis testing and confirm the intended mechanism of action of investigational agents. The panel may also be adaptable to diagnostic applications to identify patients most likely to respond to a particular apoptosis inducer.

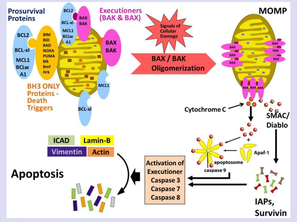


Figure 1. Intrinsic apoptosis biomarkers. Flow chart of proteins involved in intrinsic apoptosis pathway that can serve as biomarkers, and dynamic changes in various proteins during onset and culmination of apoptosis.

MOMP = Mitochondrial outer membrane permeabilization.

Materials and Methods

- The Multiplex panel includes 15 monomer and oligomeric proteins involved in intrinsic apoptosis. Sandwich immunoassays were built on the Luminex® xMAP platform using pairs of high-affinity antibodies. Measurement of the oligomeric forms of biomarkers BAK-MCL1, BAX-Bcl2, and BAK-BCL-xl was achieved by capturing BAK and BAX protein and probing with labeled antibodies specific for MCL-1, BCL-xl, and BCL2, respectively. Specificity of the antibody pairs and subsequent sandwich assays were confirmed by immunoprecipitation (IP) with capture antibodies and by Western blot (WB) with detector antibodies.
- Oligomeric forms of BCL2 family proteins were generated as covalantly linked fusion proteins with a small spacer, and used as calibrators.
- Markers were grouped into three panels (**Table 1**) to minimize artifacts in measurements due to protein–protein interactions among markers. Each panel will be available as a separate kit.

Table 1. Composition of multiplex panels

Panel 1	Panel 2	Panel 3
1. BAK, Total	1. BIM, total	1. BAK-MCL1 heterodimer
2. BAX, Total	2. BAD, total	2. BAK-BCL-xl heterodimer
3. Caspase-3, Total	3. BAX-BCL2 heterodimer	3. Caspase-3, cleaved
4. Lamin-B	4. BCL-xl, total	4. Phospho-S99BAD
5. SMAC (dimer)	5. MCL1, total	5. Survivin

- Sample-handling procedures were developed to generate cytosolic and mitochondrial (+nuclear) fractions of tissue lysates and maintain the non-covalent association of BH3 domain proteins during the assays.
- Analytical validation was performed using tumor cell lysates and included reproducibility, dilution recovery, accuracy, and sample stability studies.
- A fit-for-purpose approach was used to validate panel clinical utility. Preclinical studies were conducted using molecularly targeted drugs. Sample processing SOPs were used to process tumor tissue samples to analyze biomarkers.

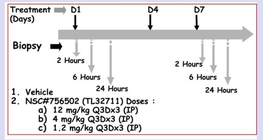


Figure 2. Schema of a preclinical study of MD-MB231 human breast cancer xenografts treated with SMAC mimetic TL32711. Xenograft tumor samples were collected after dose 1 (day 1) and dose 3 (day 7).

Results

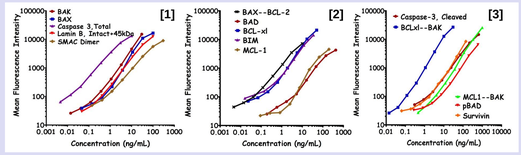
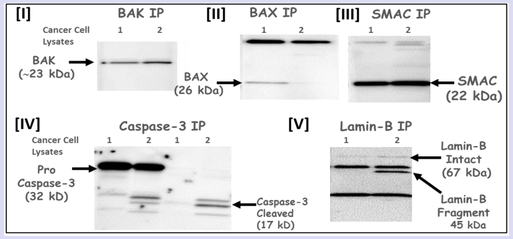


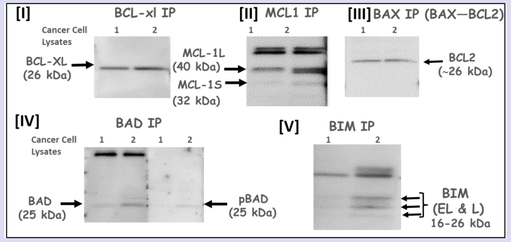
Figure 3. Calibration Curves for Multiplex Panels 1–3. Sandwich immunoassays were developed using capture and labeled antibody pairs and recombinant protein calibrators.

Specificity of Antibody Pairs Selected for Sandwich Immunoassays

Panel 1



Panel 2



Panel 3

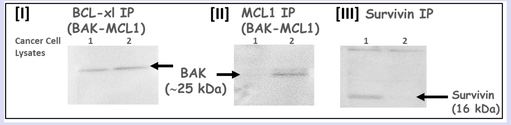


Figure 4. Specificity of capture and detector antibodies demonstrated by IP and subsequent WB (Panels 1–3). Cell or tissue lysates were immunoprecipitated using capture antibodies. IP proteins were run on SDS-PAGE and analyzed by WB using detector antibodies. These results not only demonstrate the specificity of the antibody pairs but also identify the isoforms measured by these assays. Two markers from Panel 3, pBAD and cleaved Caspase-3, are shown in Panel 2. The intensity of WB bands correlated with concentrations (details not shown) of the respective marker determined by multiplex panel in the same cell lysates, shown as samples 1 and 2.

Table 2 (A–C). Crossreactivity of Immunoassays in Multiplex Format*

A)

Single Detection	Multiplex Beads					
	BAK	BAX	Cas-3, total	Lamin B	SMAC	Sample
BAK	100%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	Calibrator Cocktail/Tumor Lysate
BAX	5% (lysate)	100%	<LLOQ	<LLOQ	<LLOQ	
Cas-3, total	<LLOQ	<LLOQ	100%	<LLOQ	<LLOQ	
Lamin B	<LLOQ	<LLOQ	<LLOQ	100%	<LLOQ	
SMAC	<LLOQ	<LLOQ	<LLOQ	<LLOQ	100%	

B)

Single Detection	Multiplex Beads					
	Cas-3. cleaved	BCL-xl -BAK	MCL-1 - BAK	pBAD	Survivin	Sample
Caspase-3, cleaved	100%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	Calibrator Cocktail/Tumor Lysate
BAK	<LLOQ	100%	100%	<LLOQ	<LLOQ	
pBAD	<LLOQ	<LLOQ	<LLOQ	100%	<LLOQ	
Survivin	<LLOQ	<LLOQ	<LLOQ	<LLOQ	100%	

C)

Single Detection	Multiplex Beads					
	BAD	BAX-BCL2	BCL-xl	BIM	MCL-1	Sample
BAD	100%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	Calibrator Cocktail/Tumor Lysate
BCL-2	<LLOQ	100%	<LLOQ	<LLOQ	<LLOQ	
BCL-xl	<LLOQ	<LLOQ	100%	<LLOQ	<LLOQ	
BIM	<LLOQ	<LLOQ	<LLOQ	100%	<LLOQ	
MCL-1	<LLOQ	<LLOQ	<LLOQ	<LLOQ	100%	

*Crossreactivity was tested using multiplex beads with either multiplex calibrators or a tumor lysate and single detections. All immunoassays were specific for the respective target except for total BAX, which showed 5% crossreactivity in tumor lysates.

Table 3 (A–C). Analytical Sensitivity of Multiplex Immunoassays.

A) Panel 1	BAK	BAX	Cas-3, Total	Lamin B	Smac/DIABLO
LLOQ (ng/mL)	0.78	0.89	0.0073	0.14	0.50
F-LOD (ng/mL)	0.89	1.1	0.0071	0.18	0.54
%CV at F-LOD	16%	17%	11%	9%	11%

B) Panel 2	BAD	BAX – BCL2	BCL-xl	BIM	MCL1
LLOQ (ng/mL)	0.52	1.9	0.089	0.046	1.2
F-LOD (ng/mL)	0.67	2.5	0.154	0.063	1.4
%CV at F-LOD	16%	16%	11%	16%	11%

C) Panel 3	BCL-xl - BAK	Cas-3, cleav.	MCL-1 - BAK	pBAD	Survivin
LLOQ (ng/mL)	0.11	0.062	1.2	2.1	0.11
F-LOD (ng/mL)	0.12	0.095	1.4	3.6	0.32
%CV at F-LOD	19%	11%	10%	9%	10%

Lower limit of quantitation (LLOQ) was defined as mean±10xSD for blank (n=20) signal. Functional limit of detection (F-LOD) was assessed using a control sample with analyte concentrations just above the LOQ value, with CV<30%.

Table 4. Reproducibility and Accuracy of Multiplex Immunoassays (selected data)

	Analyte	Precision		Sample Linearity (Recovery)	Spike Recovery
		Intra-assay (n=20)	Inter-assay (n=5)		
Panel 1	BAK	4%	8%	102%	103%
	BAX	5%	11%	83%	87%
	Caspase, Total	5%	9%	106%	112%
	Lamin-B,	4%	8%	121%	52%
	Smac/DIABLO	7%	12%	85%	80%
Panel 2	BAD	5%	9%	80%	139%
	BAX – BCL2	6%	9%	84%	90%
	BCL-xl	3%	4%	74%	104%
	BIM	3%	12%	123%	108%
	MCL-1	6%	6%	102%	76%
Panel 3	BCL-xl - BAK	5%	10%	79%	143%
	Caspase-3, Cleaved	3%	6%	125%	106%
	MCL1 - BAK	5%	10%	87%	80%
	pS99BAD	5%	7%	119%	96%
	Survivin	4%	6%	106%	132%

Precision (intra- and inter-assay) was determined by measuring 3 control samples (high, medium, and low) in replicates of 20 over 5 runs, with a minimum of 2 hours between runs. Mean CV (%) from medium control is shown. Linearity was determined by diluting a sample over 3 dilutions steps (1:2, 1:4, and 1:8) with assay buffer. Spike recovery was performed by spiking different amounts of calibrator protein into lysate samples. Recoveries were calculated as percent of non-spiked or non-diluted lysate samples; average of 4 spike levels is presented. Lamin-B consistently showed ~50% spike recovery, suggesting alternate strategies must be used to confirm accuracy.

Fit-for-Purpose Evaluation of Multiplex Panel

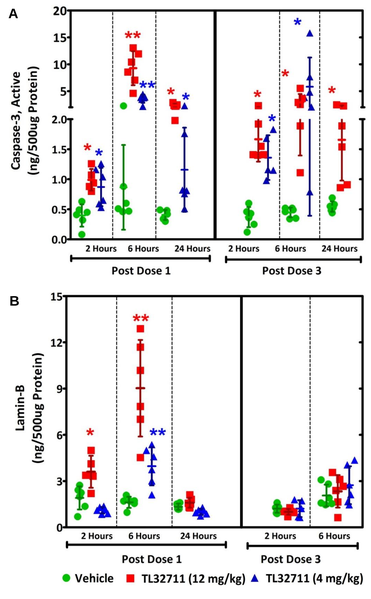


Figure 5. Changes in cleaved caspase-3 (A) and Lamin-B (B) in the cytosolic fraction of tumor xenografts treated with SMAC mimetic TL32711. Significant upregulation of cleaved caspase-3 was observed within 2 hours post-treatment. Peak differences (10- to15-fold increase) were observed at 6 hours post-treatment. Similar changes were observed in BCL-xl levels (data not shown). Analysis of Lamin-B at 24-hour time point ongoing. *p<0.05, **p<0.001 vs. vehicle

Summary and Conclusions

We describe successful development and validation of a multiplex panel of apoptosis biomarkers. Measurement of the oligomeric forms of the BCL2 family proteins by immunoassay is novel, as it evaluates a pathway based on alterations in protein dynamics rather than on absolute protein levels.

The panels demonstrated acceptable analytical performance in terms of reproducibility, accuracy, and analyte stability for quantitative measurement. Analyte molecular characteristics measured in each immunoassay in the panel were confirmed by established methods.

Sample-handling procedures (SOPs) developed in this study allow measurement of markers in cytosolic and mitochondrial (+nuclear) fractions in <100 µg protein from tumor cell lysates.

Preclinical analysis of intratumoral levels of caspase-3, Lamin-B, and BCL-xl in a breast cancer xenograft model showed the indicated expected directional changes after treatment with a SMAC mimetic, demonstrating fit-for-purpose utility of the multiplex panels.

The assays were successfully transferred (details not shown) from the developmental lab at RBM Myriad to NCI and are ready for clinical use. The assays will be available commercially from Bio-Rad as Luminex kits.

The panel will facilitate real-time preclinical and clinical evaluation of drugs targeting apoptosis.

All animals used in this research project were cared for and used humanely according to the following policies: the *U.S. Public Health Service Policy on Humane Care and Use of Animals (2000)*; the *Guide for the Care and Use of Laboratory Animals* (1996); and the *U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* (1985). All Frederick National Laboratory animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Funded by NCI Contract No HHSN261200800001E.

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