Abstract #4878

Application of a novel nano-immunoassay platform to assess changes in cIAP1 in response to the SMAC-mimetic, LCL161

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ABSTRACT

Introduction: cIAP1 (BIRC2) is member of a class of inhibitor of apoptosis proteins (IAP) that function in a regulatory role to prevent unintended cell death by apoptosis. LCL161 is a small molecule antagonist of IAPs that reverses that effect of cIAP1 causing apoptosis. The present study explores the use of the NanoPro 1000, a novel proteomic platform to measure the changes in cellular cIAP1 in response to treatment with LCL161.

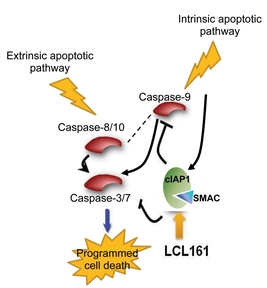
Methods: SKOV3 cells, peripheral blood mononuclear cells (PBMC) and hair follicles collected from healthy donors were treated with LCL161 for different times and subjected to capillary iso-electric focusing. Immunodetection was performed using antibodies directed against cIAP1 and the signal was quantified using HRP-labeled chemiluminescence reagents. Data from the NanoPro were compared to results from western blots run in parallel.

Results: We report that results from the nano-immunoassay are reproducible and correlate well with the western blotting data. Modulation in the cellular levels of cIAP1 in response to LCL161 treatment could be captured in samples containing as little as 1 ng of total protein. Similar modulation of cIAP1 was seen upon exposure to LCL161 in the studied test matrices.

Conclusions: This novel proteomic tool is more sensitive, specific and quantitative compared to the currently available option of western blotting assays which are labor intensive, and have a relatively low throughput. Measurable signals corresponding to cIAP1 levels can be detected in matrices like PBMCs and hair follicles. The modulation of cIAP1 levels in these matrices mirror that seen in SKOV3 cells.

INTRODUCTION

cIAP1 is a member of the inhibitor of apoptosis (IAPs) family of proteins. It is up-regulated in several human cancers and plays an important role in tumor survival. cIAP1 functions to prevent cellular apoptosis by preventing the activation and/or inhibiting the function of different caspases. The mitochondrial protein, SMAC/DIABLO, a natural antagonist of IAPs binds to cIAP1 and inhibits its anti-caspase activity leading to induction of apoptosis. Several different small molecule SMAC-mimetics have been developed over the past years as a means to sensitize cancer cells to apoptosis. LCL161 is small molecule SMAC-mimetic that antagonizes the action of cIAP1 resulting in cell death. Here we studied the effects of LCL161 on cellular cIAP1 protein using a novel nano-immunoassay technology. Existing methods to evaluate cIAP1 by either ELISA or western blotting are hampered by a lack of reagents, a need for large sample volumes and poorly quantifiable results.



Schematic view showing the apoptotic cascades and the interplay between cIAP1 and SMAC

NanoPro 1000 is a novel technology based on capillary isoelectric focusing coupled to an immunoassay. Protein isoforms are separated based on their pI, immobilized and detected by antibodies tagged with a chemiluminescent probe. This technology is able to characterize proteins in extremely small sample volumes allowing us to use non-traditional matrices such as PBMCs and hair follicles.

In an attempt to identify a method to evaluate cIAP1 that is more robust, quantitative and amenable to evaluating small volumes of clinical samples, we explored the use of NanoPro 1000.

METHODS

Sample collection: PBMC and hair samples are collected in accordance with IRB protocol for Novartis blood donor program. Informed consent was obtained from all healthy volunteers according to the guidelines.

SKOV3 cells are maintained in a humidified 5% CO_2 incubator at 37°C. Cell lines are grown in RPMI1640 medium, penicillin/streptomycin and 10% fetal bovine serum.

Sample treatment: SKOV3 cells (4x10^6/ml), blood (8 ml) and hair follicles (10-15 follicles) were treated with 2μM LCL161 or DMSO for 2hrs.

Sample preparation: Cells are harvested and lysed with Bicine CHAPs buffer with protease and phosphatase inhibitors (CellBiosciences) for 30 min on ice. Lysates were cleared by centrifugation at 13,000 x g for 15 min at 4°C and protein concentrations were determined by Lowry method (BioRad).

NANO-Immunoassay

Nano-immunoassay was carried out by separating and immobilizing the analyte by capillary isoelectric focusing followed by immunoprobing and quantitation.

Sequential steps involved in this process are as follows:

Load

The capillary is filled with a 400-nL mixture of sample, fluorescently labeled pI standards and ampholytes.

Separate

Voltage is applied across the capillary to drive the IEF separation. Individual proteins and pI standards concentrate at their isoelectric points and the position of each standard in the capillary is recorded.

Immobilize

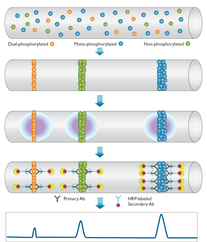
The capillary is exposed to UV light, activating the proprietary linking chemistry and locking the separated protein isoforms to the capillary wall.

Immunoprobe

The capillary is rinsed and immunoprobed for specific proteins. Luminol and Peroxide are added to catalyze the generation of chemiluminescent light, which is captured by a CCD camera.

Quantitate

The digital image is analyzed and quantitative results are presented in the Compass software.



(Taken from CellBiosciences assay development guide)

General Assay Conditions

Gradient	pI 3-10
UV Exposure (immobilization)	40 sec.
Standard ladder	1 (pI 4.0, 4.9, 6.0, 6.4, 7.3)
Separation conditions	40min, 15000uW
Primary antibody incubation	2 hr
Secondary antibody incubation	30 min
Exposure	60sec, 120sec, 240sec and 960 sec

Table 1. General assay conditions used unless stated in the legend.

RESULTS

I. Assay Optimization

Antibody screening:

A total of 3 antibodies against cIAP1 were tested. Two out of three antibodies could detect a peak at pI 5.81, which matches the theoretical pI of cIAP1. The main criteria for antibody selection was reproducibility of the peak profiles and signal to noise ratio. We selected AF8181 from R&D systems based on the criteria for antibody selection.

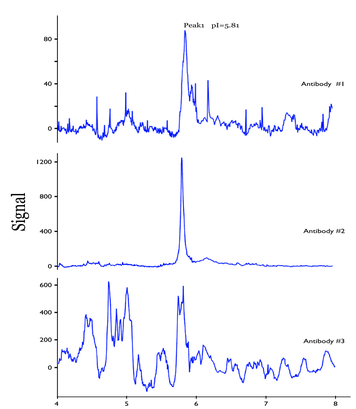


Figure 1: cIAP1 Antibody screening
Bicine/CHAPS lysate from untreated SKOV3 cells (0.2mg/ml in the capillary) was separated according to assay conditions in table 1, and probed with the indicated antibodies. Antibody-1:α- cIAP1 (Enzo life sciences ALX-803-335) 1:20 dil, Antibody-2: α-CIAP1 (R&D systems, AF8181) 1:10 dil, Antibody-3: α-cIAP1 (In house monoclonal Antibody) 1:5 dil. While all three antibodies recognized a specific peak, the signal strength for antibody 2 was the strongest.

Immobilization:

Following separation, proteins are immobilized to the coated capillary wall by UV light. Longer exposure may damage the proteins, while shorter exposure may not complete the immobilization process. Hence, optimal exposure time must be identified for each target protein of interest. The optimal exposure time for cIAP1 was determined to be 40 sec.

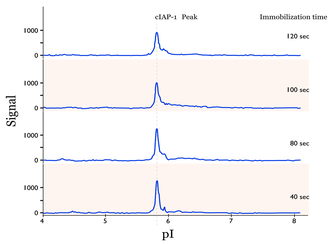


Figure 2: UV titration
SKOV3 cell lysate was diluted to a final concentration of 0.2 mg/ml in the capillary. Sample in the capillaries were exposed to UV light for different lengths of time as indicated above and probed with anti-cIAP1 (AF8181 from R&D systems, 1:10 dilution). All other assay conditions were as described in Table 1.

Antibody titration:

Titration of primary and secondary antibody conditions must be optimized to reach the binding saturation. The cIAP1 antibody was tested at various dilutions to achieve optimal signal strength. We varied primary antibody dilutions between 1:10 – 1:100 while the secondary antibody was used at a dilution of 1:100.

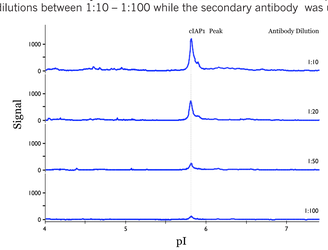


Figure 3: Primary antibody titration
SKOV3 cells were lysed with Bicine/CHAPS buffer and diluted to a final concentration of 0.2 mg/ml in the capillary. Samples in the capillary were probed with cIAP1 antibody at dilutions stated above. All other conditions as described in Table 1.

Sample protein concentration

To determine the optimal protein concentration for detection of cIAP1, samples were diluted and analyzed. A final protein concentration of 0.2 mg/ml in the capillary was found to be optimal for SKOV3. An increase in the cIAP1 signal strength was observed with increasing protein concentration, but the increase in the signal strength was found to be non-linear.

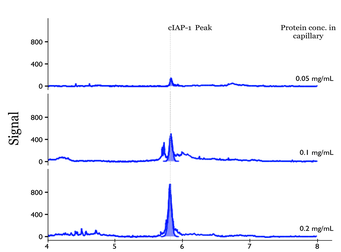


Figure 4: Sample protein concentration
SKOV3 cells were lysed with Bicine/CHAPS buffer and diluted to different final concentration as indicated above. The samples were analyzed as described in Table 1. Similarly, a final protein concentration of 0.25mg/ml was found to be optimal for PBMCs (data not shown).

2. cIAP1 in different matrices

To determine if the optimized assay conditions are applicable to different matrices, cIAp1 was analyzed in SKOV3 cells, PBMCs and hair follicle cells. cIAP1 was detectable in all the three matrices under the tested conditions at varying signal strengths reflecting either different endogenous cIAP1 concentrations, and/or a need for different optimal assay conditions for each matrix.

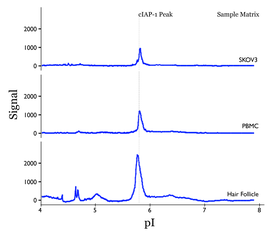


Figure 5: cIAP1 in different matrices
PBMCs and hair follicles from healthy donors were collected and lysed with Bicine/CHAPS buffer and diluted to final concentration of 0.25 mg/ml in the capillary and probed as described in Table 1.

3. cIAP1 signal specificity

To determine the specificity of the cIAP1 signal observed, purified recombinant cIAP1 protein was analyzed under the optimized assay conditions. A single peak around pI 5.89 was observed, which is similar though not identical to the peak observed with endogenous cIAP1.

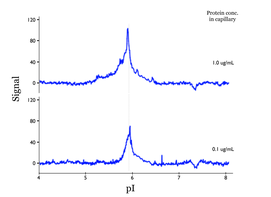


Figure 6A: cIAP1 recombinant protein
Different concentrations of recombinant cIAP1 as indicated above were analyzed as described in Table 1.

To determine if the cIAP1 signal is modulated by IAP inhibitors, PBMCs and SKOV3 cells were treated with LCL161 (2uM, for 2 hrs). The treated and untreated samples were probed with anti-cIAP1. A loss in the cIAP1 signal was observed in the LCL161 treated samples reflecting what was observed in western blots (data not shown).

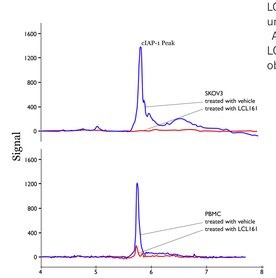


Figure 6B: LCL161 on cIAP1 levels SkOV3 cells and blood from healthy donors were treated as described in the Methods and analyzed as described in Table 1.

CONCLUSION

- cIAP1 assay conditions were optimized for protein immobilization time, primary antibody dilution, and sample protein concentration.
- Similar cIAP1 signals were identified with recombinant cIAP1 and endogenous cIAP1 from SKOV3 cells, PBMCs, and hair follicle cells.
- Modulation of cellular cIAP1 by SMAC-mimetic, LCL161, as detected by NanoPro 1000 was consistent with data from western blotting.
- NanoPro 1000 allowed the analysis of cIAP 1 from less than a thousandth of the sample required for western blot analysis.
- NanoPro 1000 facilitated increased throughput by allowing analysis of 96 samples in a single assay run.

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