Fragment Screening using Capillary Electrophoresis to measure Protein-Protein Interactions

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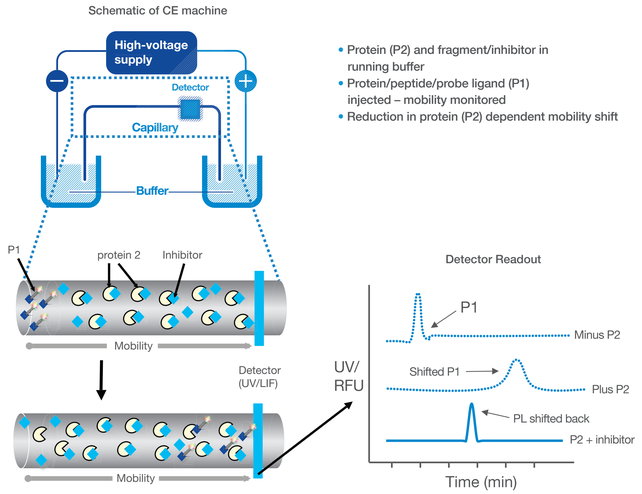
Introduction

Capillary electrophoresis (CE) has wide applications as a screening tool in the field of drug discovery (Figure 1)[1-4]. One such application is CEfrag™, a novel, proprietary technique used for fragment-based screening[5]. The technique is a microscale binding assay, ideally suited for detecting weak binding interactions between fragments and protein targets. CE is used to detect a reduction in the interaction of a probe ligand (which can be a small molecule, peptide or protein) with its target protein in the presence of a binding fragment. The competitive nature of this interaction ensures that fragment "hits" bind in a defined manner and avoids the problem of false positives caused by non-specific binding. The technique is broadly applicable to a wide range of targets, including challenging protein / peptide-protein interactions, with the ability to detect weak affinity inhibitors and with low consumption of protein. There is no need to immobilise or modify the target protein. In addition, the CEfrag™ screen can be adapted to screen for fragments and other compounds that modulate protein-protein, protein-peptide and protein-nucleic acid interactions.

Protein-protein and protein-peptide interactions are illustrated by the XIAP/SMAC assay. Two case studies of fragment screens against two different targets, Heat shock protein 90 and MDM2/p53 using the Selcia Fragment Library are also presented here. CEfrag™ hit compounds were evaluated using orthogonal assays.

Figure 1: Inhibition of protein-protein/peptide/probe ligand Interactions



- Protein (P2) and fragment/inhibitor in running buffer
- Protein/peptide/probe ligand (P1) injected – mobility monitored
- Reduction in protein (P2) dependent mobility shift

XIAP/SMAC

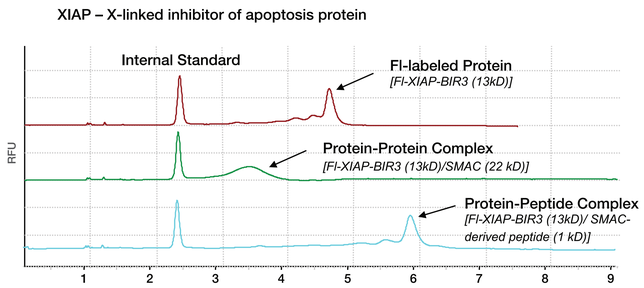
Programmed cell death is regulated by Inhibitors of Apoptosis Protein (IAP) which inhibit the caspase family of enzymes. Targeting IAPs such as X-linked IAP (XIAP) may be a way to treat cancers that overexpress IAPs such as prostrate and lung cancer and hence confers resistance to chemotherapy. Endogenous antagonists e.g. second mitochondria-derived activator of capases (SMAC protein) can bind to IAPs and promote caspase activation. Similarly small peptides (SMAC derived peptide) can also bind to XIAP, mimicking the SMAC activity.

A XIAP/SMAC assay was developed using fluorescently labelled XIAP protein (FI-XIAP-BIR3; Figure 2). XIAP-BIR3 domain binds to and inhibits caspase 9. A mobility shift was observed upon binding of SMAC protein. Similarly a SMAC peptide mimetic interacting with XIAP was also observed, demonstrating the potential of this assay to screen for SMAC peptide mimetics.

Method:

Assay development was performed on a Beckman P/ACE™ MDQ CE machine. FI-XIAP-BIR3 (S-labelled), was used as a probe ligand.
Conditions: coated capillaries 50µm i.d. x 375µm o.d. (Polymicro technologies); Injection buffer (100µl): TrisBis buffer, (150mM, pH 7.2, 2mg/ml BSA) + FI-XIAP-BIR3 (50nM); Running buffer (200µl): TrisBis buffer (150mM, pH 7.2, 2mg/ml BSA), ±SMAC Protein or ±SMAC peptide (ARPFAQKSE) (50nM); Injection: 0.5 psi for 10 sec; separation: 15kV, Reverse Polarity (cathode to anode).

Figure 2: Protein-Protein and Protein-Peptide Binding Interactions – XIAP/SMAC



- FI-XIAP-BIR3 Fluorescent protein + internal standard injected.
- CE assay detects XIAP/SMAC and XIAP/SMAC peptide mimetics interaction

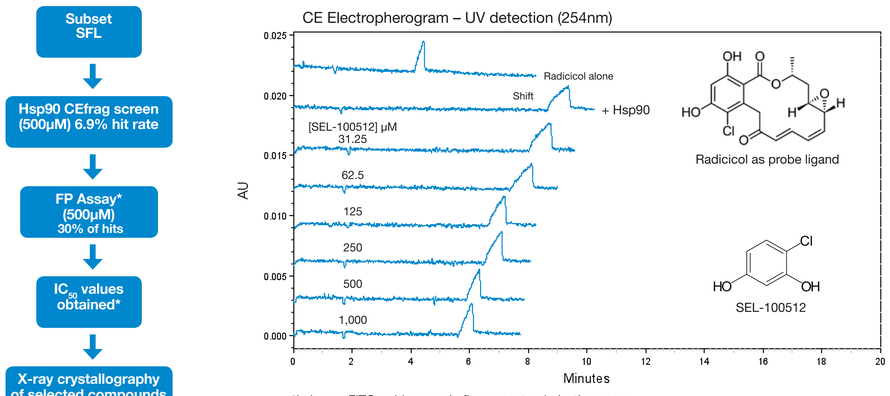
Hsp90 CEfrag™

Heat shock protein 90 is a molecular chaperone, which is up regulated in response to stress. It is involved in protein folding, cell signalling and oncogenesis; hence is a therapeutic target for oncology. Fragment screening has successfully been performed using other fragment screening techniques[6,7] with approaches leading to Hsp90 inhibitors now in clinical development[7]. A Hsp90 CEfrag™ assay was developed using radicicol as a probe ligand. A subset of the Selcia Fragment Library was screened.

Method: assay development was performed on a Beckman P/ACE™ MDQ CE machine and screening was performed on a Gemini (4-capillary instrument)[8]. Radicicol (IC_{50} 23nM, Tocris), which binds to the ATP binding pocket, was used as a probe ligand. Conditions: uncoated capillaries 50µm i.d. x 375µm o.d. (Polymicro technologies); injection buffer (100µl): Tris-Tween (10mM Tris buffer, pH 7.5 containing 0.0005% Tween and 5mM MgCl2) + radicicol (150µM) ± inhibitor or DMSO; running buffer (200µl): Tris-Tween ± inhibitor or DMSO ± Hsp90α (Assay Designs) (0.25µM); injection: 0.5 psi for 5 sec; separation: 15kV, Reverse Polarity (cathode to anode).

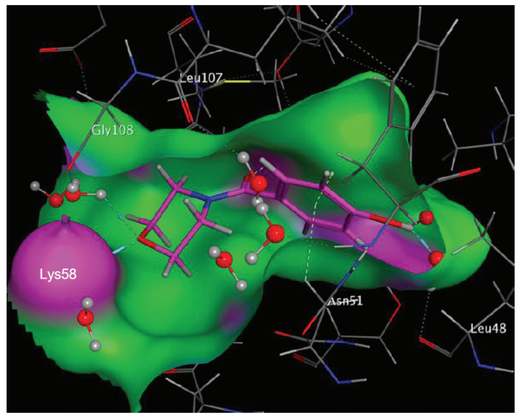
A subset of the Selcia Fragment Library (609 compounds) was screened at 500µM (f/c 1.67% DMSO) and all hits were confirmed by rescreening. The hits were scored as strong (>50% inhibition) or weak (~20-50% inhibition). All primary hits were screened at 500µM in a fluorescence polarisation (FP) competition assay using FITC-geldanamycin (K_d 23nM, StressMarq)[9]. Selected fragments were cocrystallised with N terminal Hsp90α (Figure 4).

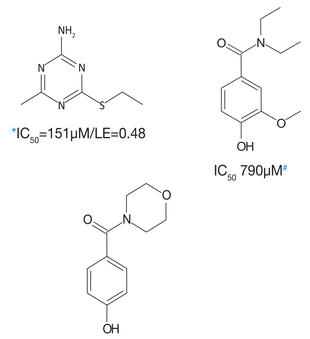
Figure 3: Hsp90 CEfrag™ Assay Development Inhibition of radicicol/Hsp90 Interaction by Fragment SEL-100512



*In house FITC-geldanamycin fluorescent polarisation assay

Figure 4: Hsp90 CEfrag™ X-ray Crystal Structures Obtained





#ITC value from literature[6]
*In house FITC-geldanamycin fluorescent polarisation assay

Conclusion

Selcia have developed a novel method to detect weak fragment binding interactions with soluble protein targets using capillary electrophoresis. This proprietary technique provides a useful tool in the fragment screening tool box, especially for challenging target classes such as protein-protein and protein-peptide interactions. It can be used as a primary screening tool (CEfrag™ screen) using either the Selcia Fragment Library or customers library; alternatively it can be used as an orthogonal technique for confirming fragment hits obtained by other methods.

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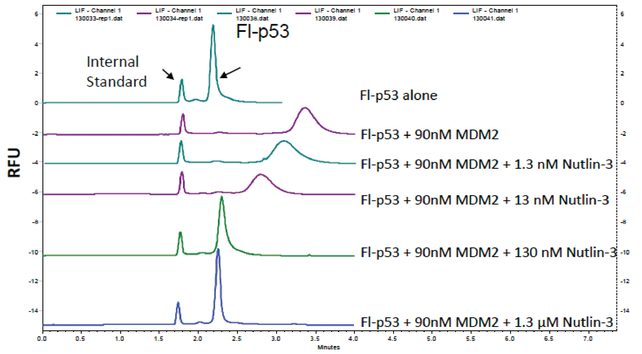
MDM2 CEfrag™

MDM2 is an important negative regulator of the p53 tumour suppressor. MDM2 protein functions both as an E3 ubiquitin ligase that recognizes the N-terminal trans-activation domain (TAD) of the p53 tumour suppressor and acts as an inhibitor of p53 transcriptional activation. Several human tumour types have been shown to have increased levels of MDM2, including soft tissue sarcomas and osteosarcomas as well as breast tumors supporting the role of MDM2 as an oncogene and validating it as an important therapeutic target for oncology.

The discovery and design of compounds able to restore mutated p53, a decade ago deemed to be impossible, has now become an attractive research field. This is due to recent fragment screening showing several co-crystal structures of small molecules in specific binding sites of mutated p53. MDM2 antagonists and p53 restoring agent are a rapidly emerging drug discovery area, in a broader sense comparable to the status of the kinase field 15 years ago. A MDM2 CEfrag™ assay has been developed at Selcia, utilising fluorescent p53 peptide as a probe ligand and selected fragments from the Selcia Fragment Library have been screened

Method: assay development was performed on a Beckman P/ACE™ MDQ CE machine and screening was performed on a Gemini (4-capillary instrument)[8] , both with LIF detection. Fluorescent p53 peptide was used as a probe ligand. Conditions: coated capillaries 50µm i.d. x 375µm o.d. (Polymicro technologies); injection buffer (100µl): 50 mM TAPS/TRIS, pH 8 buffer, pH 7.5 containing 0.01% Tween 20 and 0.1 mM DTT + fluorescent p53 peptide (300 pM) + 20 pM fluorescein (internal standard) ± inhibitor or DMSO; running buffer (200µl): 200 mM TAPS/TRIS, pH 8 buffer, pH 7.5 containing 0.01% Tween 20 and 0.1 mM DTT ± inhibitor or DMSO ± MDM2 (N-terminal); injection: 0.5 psi for 5 sec; separation: 10 kV, Reverse Polarity (cathode to anode).

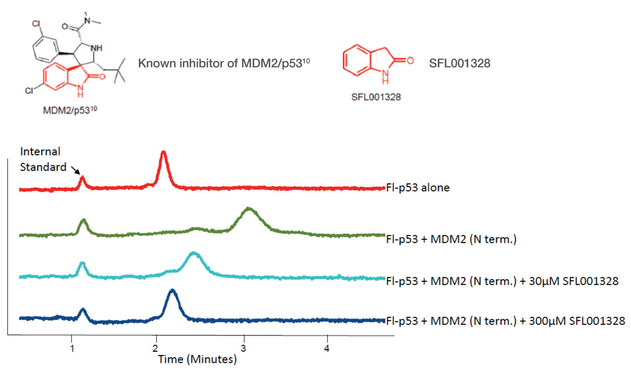
Figure 5: MDM2/p53 – Inhibition by Nutlin-3



MDM2 Ubiquitin Ligase (Protein) and p53 (Peptide), an interaction involved in cancer, form an easily detectable complex disrupted by inhibitor Nutlin-3.

Measured: IC50~13nM

Figure 6: Protein Peptide Interactions: CE Assay Detects Weak Fragment Inhibitor of MDM2/p53



- FL-p53 injected, N terminal MDM2 and fragment in running buffer.
- Small subunit of a known potent inhibitor, oxindole SFL001328 (Mwt 133), disrupts interaction between N terminal MDM2 (Protein)- FL-p53 (Peptide).
- Confirmed as hit by Saturation Transfer Difference NMR