original research report

# Serine and proline-rich ligands enriched via phage-display technology show preferential binding to BCR/ABL expressing cells

Karen Shires [a,b,*], Iva Shankland [a], Shaheen Mowla [b], Samuel Njikan [b], Jai Jaymacker [b], Nicolas Novitzky [b]

[a] National Health Laboratory Services (NHLS)/Groote Schuur Hospital, Haematology, Cape Town, South Africa, [b] Division of Haematology, University of Cape Town, Cape Town, South Africa

[*] Corresponding author at: Division of Haematology, Department of Clinical Laboratory, Services, University of Cape Town, Observatory, Cape Town, South Africa. Tel.: +27 723476558 · karen.shires@uct.ac.za · Accepted for publication 4 January 2014

Hematol Oncol Stem Cell Ther 2014; 7(1): 32–40


DOI: http://dx.doi.org/10.1016/j.hemonc.2014.01.001

**BACKGROUND AND OBJECTIVES:** Despite the use of targeted therapy, chronic myelogenous leukemia (CML) currently remains incurable with drug therapy, with patients requiring life-long treatment. Developing either a vaccine to prevent the disease or another novel drug to specifically target and eradicate the CML cell will require the identification of CML-associated cell-surface markers and molecules that can bind specifically to the cell surface. In an attempt to discover peptides that bind specifically to cells in the early chronic phase of the disease, we used phage-display technology to identify heptapeptides that bind specifically to the surface of BCR/ABL-expressing fibroblasts.

**METHODS:** An *in vitro* system using NIH3T3 stably transfected with pGD210 (BCR/ABL) was used as a model for the chronic phase of the disease. The cells were panned using a linear heptapeptide phage library (Ph.D 7.0) in a negative/positive panning strategy with NIH3T3 containing only the plasmid vector as the wild type control.

**RESULTS:** We identified four novel peptides that were enriched through this technique. These peptides contained either multiple proline residues or serine/threonine–proline pairs and showed a confirmed binding preference for BCR/ABL+ fibroblasts. The peptide Y-R-A-P-W-P-P also showed a binding affinity for granulocytes from untreated CML patients.

**CONCLUSION:** We have identified several novel peptides that can be used in future studies to identify specific CML cell-surface antigens or provide a novel drug-delivery mechanism.

Although CML is one of the best described leukemic diseases, the only definitive cure continues to be bone marrow transplants. Treatment with specific tyrosine kinase inhibitors (TKI) offers a targeted therapy approach and is currently the standard of care for chronic phase CML, yet in most cases life-long therapy is required to prevent a relapse. In addition, despite remission being rapidly achieved with TKI therapy in many patients, some do not respond at all, suffer severe side-effects, or acquire resistance mutations and relapse while on therapy.[1,2] Alternative therapies, including the development of a CML vaccine[3–5] and targeted drug-delivery mechanisms,[6] both of which are based on the concept of a unique CML or leukemic cell-surface antigen, are thus currently being investigated. Although BCR/ABL, the novel fusion protein responsible for chronic phase CML development, can be processed and presented on the surface of clonal cells, it is not highly immunogenic and has shown limited success in vaccine trials with patients in the chronic phase.[7–9] Despite the use of global microarray techniques[10] and cancer/testis antigen profiling,[11] CML cell-surface antigens remain poorly defined. Research is thus still ongoing to identify a CML-specific antigen which holds potential for the development of a vaccine and targeted drug-delivery.

Phage-display technology, using either peptide or antibody libraries to identify ligands and receptors, is a technique that is proving useful in the search for cancer-specific markers.[12–14] Despite the technical difficulties associated with whole-cell panning (i.e.,

low level targets, suitable negative control cell types), in the arena of haematological malignancies, this technique has already generated useful targets. This includes potentially new drug-delivery mechanisms in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL),[6] identification of growth and proliferation modifying ligands in AML[15] and potential diagnostic markers for IFN-α sensitive CML cells.[16]

In the search for a CML-specific antigen, we used phage-display technology to identify peptides that bind to the surface of BCR/ABL expressing cells, as a means of identifying ligands that are associated specifically with chronic phase CML disease. Due to the lack of suitable human cell-lines that represent chronic phase disease and normal physiology, and the difficulty associated with stable transfection of human haematopoietic stem cells,[17] we chose to use a BCR/ABL-expressing NIH3T3 mouse fibroblast cell-line as a model. Mouse and rat fibroblast models have been used extensively in defining the pathogenic role of BCR/ABL[18–21] and allow for the development of a well-controlled, genetically defined system for the whole-cell panning phage-display approach. We generated stably transfected clones using the *pGD210* BCR/ABL expression vector,[22] as well as a modified vector-only control (*pCVT*). These cells were assessed for cell-surface associated changes previously attributed to BCR/ABL expression and then panned using a linear heptapeptide phage library in a negative/positive panning approach to identify peptides that bind preferentially to BCR/ABL expressing cells.

## MATERIALS AND METHODS

### Stable clone generation

The BCR/ABL expression vector p*GD210*,[22] (kindly supplied by Prof G. Daley) was used to generate the p*CVT* control plasmid, by excising the 7.2 kb *bcr/abl* cDNA fragment with *XhoI* and re-ligating the vector. Endotoxin-free plasmid DNA (4 μg p*GD210* and 2 μg p*CVT*) (Nucleobond Xtra EF, Macherey–Nagel) was used to transfect NIH3T3 mouse fibroblasts ($2 \times 10^6$) using Nucleofector technology (LONZA, program U-023). Transfected NIH3T3 were maintained in RPMI/10% FBS media (Gibco) and 400 μg/ml G418 (Promega) antibiotic selection was started 48 h post-transfection for a 14-day period to select transfected clones. Surviving clones were expanded in the presence of G418, and cryopreserved for later use.

### BCR/ABL mRNA expression

Ribonucleic acid (RNA) was extracted using Total RNA isolation reagent (TRIR) (ABgene) and the levels of BCR/ABL mRNA expression were determined using standardised methodology (1 μg RNA) for minimal residual disease monitoring of this transcript,[23–25] by the NHLS diagnostic service (Groote Schuur Hospital, Cape Town).

### Adherence independent growth

*Agar clonogenic assay*

Fibroblasts ($3 \times 10^5$) were mixed with 3 ml 0.3% agar/RPMI/10% FBS and plated on top of a solidified layer of 0.6% agar/RPMI/10% FBS in six well plates (Corning). Cells were incubated for 21 days and colonies visualised using phase-contrast microscopy.

*Methylcellulose assay*

Fibroblasts ($5 \times 10^3$) were mixed with 100ul 1.5% methylcellulose (Sigma–Aldrich) RPMI/10% FBS media and plated into polyHEMA (Sigma–Aldrich) coated 96-well plates. Following incubation for the specified time periods, the colony expansion was measured by the addition of 20 μl CellTiter 96 $AQ_{ueos}$ One reagent (Promega), incubation for 2 h at 37 °C/5% $CO_2$ and spectrophometry at 490 nM (Anthos2001 plate reader). Triplicate blank readings were subtracted.

### *In vitro* cell motility assay

Confluent cell layers were established in six well plates in RPM1/10% FBS and a linear wound was created by scratching through the monolayer using a sterile 200 μl pipette tip. To remove cell debris, the growth medium was replaced and mitomycin C (10 μg/ml) (Sigma–Aldrich) was added to prevent further cell proliferation. Cells were incubated at 37 °C/5% $CO_2$ and the wound width was measured at three reference points at 0 and 6 h post treatment using light microscopy and the distance migrated recorded (μm).

### Phage-display analysis

The phage-display linear heptapeptide library Ph.D 7.0 (New England Biosciences) with a complexity of $10^9$ sequences, was used to pan NIH3T3 clones, using a combination of negative/positive panning in each round.[26] Each panning round was performed using cells at P3 post-thaw (to ensure similar membrane composition) and both 3T3B.p*GD210* and 3T3C.p*CVT* cells were of the same passage following transfection. Cells ($3 \times 10^6$) were plated in 10 cm dishes, left to adhere O/N and then prewashed with