Developmental Awards Candidate Leukemia Spore


# Wadih Arap, M. D., Ph. D.

*Professor*

## Department of Genitourinary Medical Oncology

**Identification of Therapeutic Targets for Leukemia by Phage Display Profiling of Leukemia Cell Lines and Patient-Derived Samples**


## Abstract:

Despite recent advancements made to the systemic therapy of leukemia, non-specific cytotoxicity and drug resistance remain a problem resulting in long-term side effects, significant relapse and mortality rates. Targeted drug delivery by using vehicles that can preferentially home to leukemia cells offers a platform for the development of potentially more efficient and safer therapies. One approach is to target $\beta_2$ integrin–mediated cell adhesion in leukemia. The inhibitory effect of the double disulfide–restrained LLG-C4 peptide (ADGACPCFLLGCCGA) on the activated form of the $\beta_2$ integrin receptor was determined by using phage display screening. We reasoned that: (i) disease progression can be attenuated by controlling leukemia cell adhesion to the extracellular matrix, and (ii) phage display profiling of leukemia cell lines and of patient-derived samples would allow for the identification of novel therapeutic targets. Our specific objectives are: (1) to validate the LLG-C4 phage binding on leukemia-derived cell lines and patient samples, (2) to evaluate the therapeutic efficacy of the LLG-C4 peptide *in vitro* and *in vivo*, (3) to isolate other peptide motifs (ligands) that preferentially bind to cell surface markers, (4) to identify the corresponding receptors and assess interaction mechanisms, and (5) to evaluate the lead systems for targeted therapy in leukemia. We found that the LLG-C4 peptide binds to AML-derived cell lines and to the leukocyte population isolated from blood of AML patients with no significant binding to control leukocytes from healthy donors. In an *in vivo* leukemia model, the LLG-C4 peptide significantly improved the survival of leukemia-bearing mice with human OCI-AML3 cells (*P=0.018*). The peptide prevented the attachment and proliferation of the leukemia cells on growth-supporting mesenchymal cell layer. Given this proof-of-concept, we have also screened for a new set of anti-leukemia peptides. By using a phage display-based aqueous-organic phase separation (Biopanning and Rapid Analysis of Selective Interactive Ligands; BRASIL), we profiled 9 human leukemia cell lines including those derived from the NCI-60 cell panel. Screening of the MOLT-4 ALL cell line with $CX_7C$ random phage library yielded several peptide motifs of which three clones with cyclic insert sequences exhibited high frequency binding to various leukemia cell lines and to bone marrow samples derived from 6 AML and 7 ALL patients. Comparing selected motifs with sequences in on-line protein databases, we identified a number of candidate ligands that share homology with these peptides. Relative to control insertless phage, one of the clones showed a 52.27 ± 6.80 (mean ± SEM) fold higher binding to recombinant neuropilin-1 (NRP-1), a receptor for $VEGF_{165}$, as compared to 1.90 ± 0.67 fold binding to an unrelated recombinant control receptor (*P=0.011*). Since NRP-1 plays a role in leukemia, work is underway to determine whether the synthetic ligand peptide exhibits similar binding to NRP-1 and whether it has any functional effect on leukemia cells. If so, we will validate the efficacy of this peptide in mouse models of leukemia. Together, these findings will have clinical implications in that the newly identified peptide may serve as a peptidomimetic drug lead and can be optimized as a delivery vehicle for targeted therapy of leukemia.

**Background:**
Despite the advancements that have been made to the conventional systemic therapy of leukemia, drug resistance and non-specific cytotoxicity often result in adverse, long-term, side effects and significant relapse and mortality rates. In recent years, much attention has been focused on the development of targeted therapy for leukemia (Ravandi F et al., 2003). Recently, our group developed a new technology termed Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL) that would allow for selective, single-cell targeting with genetically-modified phage libraries whereby filamentous phage display random peptides on their surface fused to the phage minor coat protein pIII. Our method comprises a simple procedure that allows cell-phage complexes to be separated from the remaining unbound phage in a single step (Giordano R et al., 2001). BRASIL has been successfully used to isolate phage that selectively targeted activated endothelial cells and tumor cells. In *ex vivo/in vivo* - based strategies, we have also used BRASIL to isolate phage that homes selectively to the bone marrow (unpublished data). We reasoned that profiling of leukemia cell lines and patient-derived samples would allow for the identification of novel therapeutic targets in leukemia. Our Developmental Research Proposal for 2003 – 2004 was based on the observation that β2-integrin – mediated cell adhesion in the setting of leukemia can be blocked by the compact disulfide–restrained LLG-C4 peptide. Despite the promising preliminary data, we screened for a new set of anti-leukemia peptides due to the difficult chemical properties and poor solubility of the originally tested motif.

**Specific Aims:**
1) Isolate peptide motifs (ligands) that preferentially bind to surface markers on leukemia cell lines and patient-derived samples.
2) Identify the corresponding receptors and assess the ligand – receptor pair interaction mechanisms.
3) Evaluate the lead pairs in a leukemia mouse model for the development of targeted therapy in leukemia.

**Experimental Approach and Preliminary Results:**
For specific aim 1, we used BRASIL to identify peptide sequences that preferentially bind to leukemia cells by incubating the cells with either the $CX_5C$ or $CX_7C$ random phage libraries, or with the insert-less control phage (Fd-tet). Phage bound to the cells was recovered, and quantified. BRASIL was performed on nine leukemia cell lines including MOLT-4, CCRF-CEM, SR, RPMI-8226, K-562, and HL-60 from the NCI-60 cell panel (http://dtpws4.ncifcrf.gov). Biopanning on MOLT-4 ALL cell line with $CX_7C$ random phage library yielded several peptide motifs of which 3 clones exhibited high frequency binding to various leukemia cell lines (Figure 1) and to bone marrow samples derived from 6 AML and 7 ALL patients (Figure 2).

We then performed phage internalization assays on K-562 cells to determine if the sequence inserts in Molt-4 phage clones are capable of receptor-mediated internalization. This would be indicative of whether these motifs have the potential to deliver drugs or apoptotic moieties into leukemia cells (Figure 3). Furthermore, by comparison of the selected motifs with available sequences in on-line protein

databases, we identified a number of proteins (ligands) that share homologous sequences with these peptides. We then used biochemical methods to perform binding assays on a number of candidate receptors. Interestingly, relative to control insertless phage (Figure 4), one of the clones showed a 52.27 ± 6.80 (average ± SEM) – fold higher binding to recombinant neuropilin-1 (NRP-1), a receptor for VEGF-$_{165}$ (Soker S et al., 1998), as compared to 1.90 ± 0.67 – fold binding to an unrelated recombinant receptor (*P=0.011*).

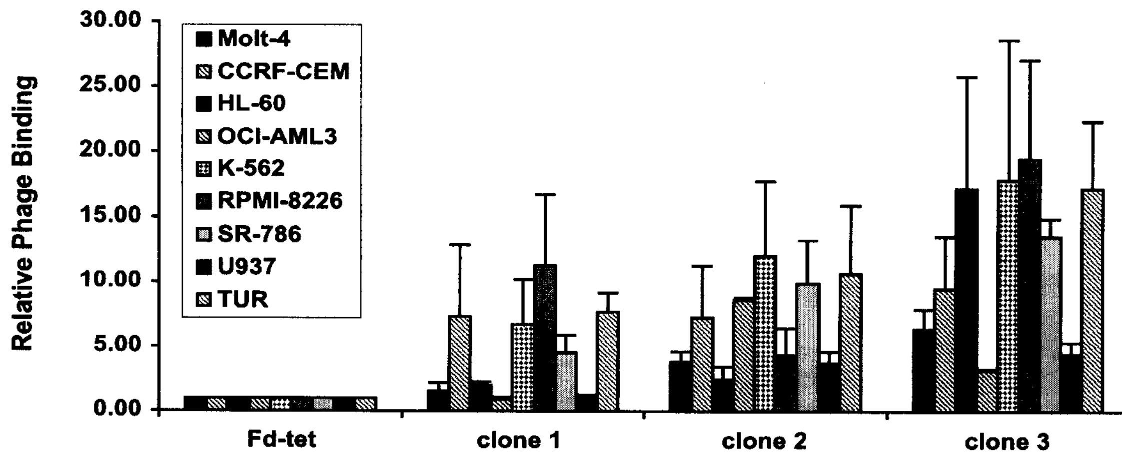


**Figure 1:** Molt-4 phage clones exhibit a variable and a strong binding pattern to a panel of leukemia cell lines including ALL; T-lymphoblast (pink), AML and CML (green), and B-lymphoblast (orange). The U937 monocytic leukemia cell line and its TPA (12-O-tetradecanoylphorbol-13-acetate) - resistant derivative TUR are indicated in blue.

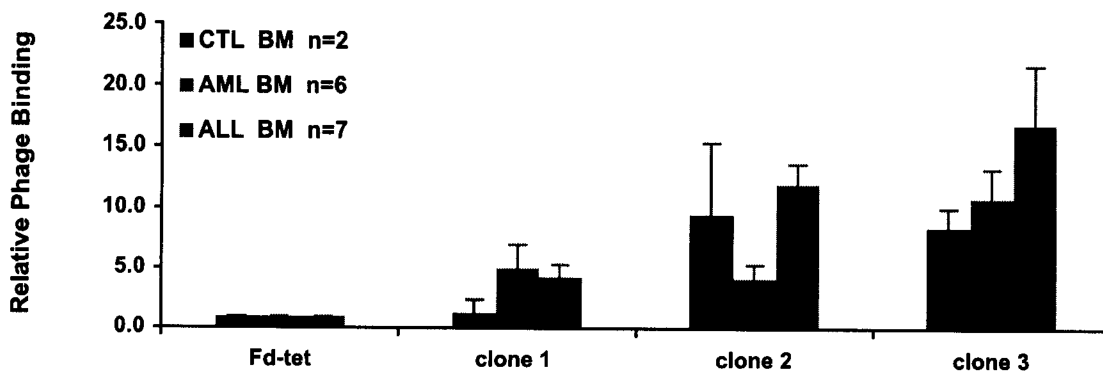


**Figure 2:** Post-normalization to Fd-tet insertless phage, binding of Molt-4 clone 1 to bone marrow (BM) cells derived from AML and ALL patients is significantly higher than BM from control (CTL) donors. The binding pattern of clone 2 suggests that its receptor could be downregulated in AML whereas the receptor for clone 3 is likely over-expressed in ALL..

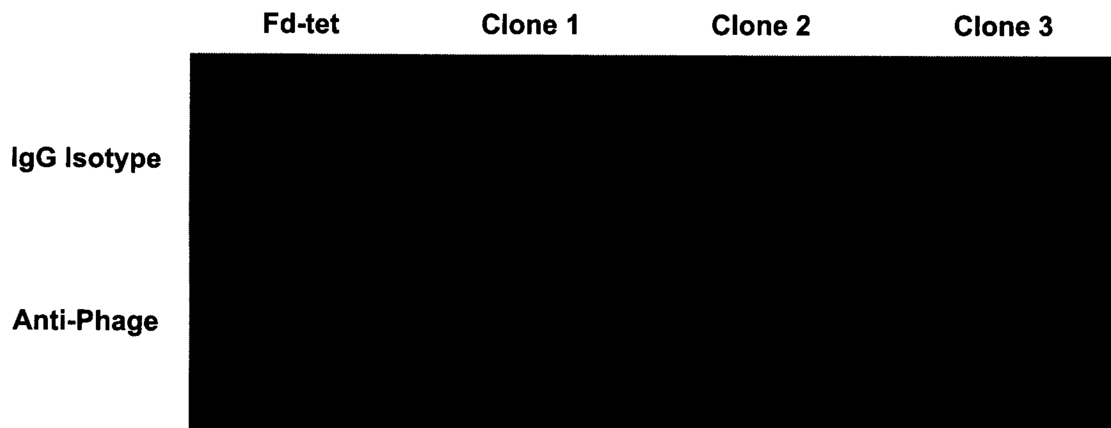


**Figure 3:** Internalization assay of Molt-4 phage clones in K-562 CML cells. The results suggest that the receptors corresponding to clones 1 and 2 are capable of internalizing the phage with clone 1 exhibiting a higher potential for receptor-mediated internalization in K-562 cells. By contrast, the receptor for clone 3 does not seem to mediate efficient internalization as indicated from the negative FITC staining of anti-phage relative to the IgG isotype control. The blue color indicates the DAPI nuclear stain.

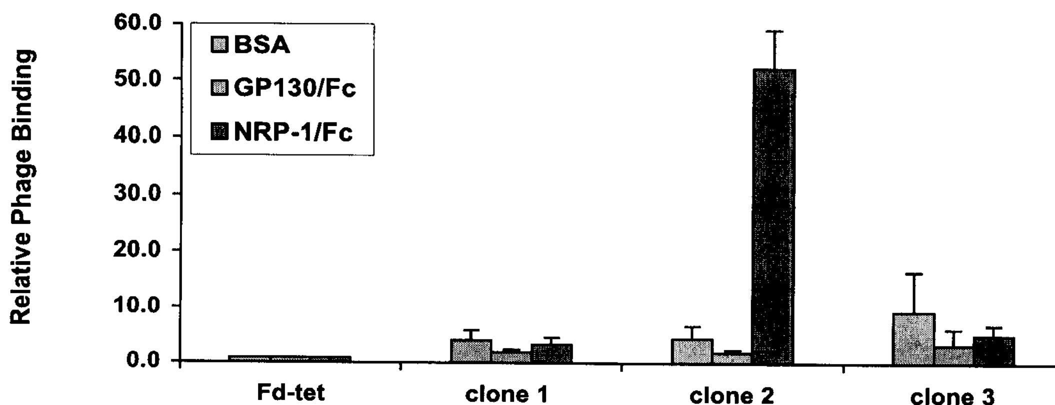


**Figure 4:** Validation of neuropilin-1 (NRP-1), a putative receptor for Molt-4 phage clones as suggested from BLAST analysis. In Binding assays, Molt-4 phage clone 2 showed a strikingly strong binding to recombinant rat neuropilin-1/Fc chimera (rrNRP-1/Fc). Relative to control insert-less phage, clone 2 had a 52.27 ± 6.80 (average ± SEM) – fold higher binding to rrNRP-1/Fc as compared to 1.90 ± 0.67 – fold binding to GP130/Fc, an unrelated recombinant receptor (*P=0.011*). The binding was specific since Molt-4 phage clones 1 and 3 showed no significant binding to either recombinant receptor. These results indicate that NRP-1 is the receptor recognized by clone 2 on the surface of leukemia cells.

**Future Directions:**
Neuropilin-1 has been shown to play a critical role in angiogenesis and its overexpression has been linked to tumor progression and aggressiveness in many cancers including leukemia (Latil A et al., 2000; Schuch G et al., 2002). Work is underway to determine if the synthetic peptide corresponding to the cyclic insert in Molt-4 phage clone 2 exhibits similar strong binding to NRP-1. We will perform binding and competition assays with related and control synthetic and recombinant peptides to explore the ligand-receptor interaction mechanisms.

We will also carry out a number of assays to test if the pure cyclic form of the peptide has any biological function on leukemia cells *in vitro*. A $CX_7C$-GG-KLAKLAKKLAKLAK-$NH_2$ conjugated form of the peptide will also be used to assess its anti-leukemia effect via delivery of an apoptotic moiety. The *in vitro* assays with the NRP-1-targeting peptides will be performed on at least 4 leukemia cell lines and will likely include: 1) cell viability and cytotoxicity assays to determine the proportion of live and dead cells in the tested populations; 2) cell proliferation assays to assess the growth rate and density of a tested cell population based on monitoring changes in total nucleic acid content, an assay that can also be adapted to quantitate adhesion of leukemia cells to bone marrow mesenchymal cell layer; 3) apoptosis assays based on detection of nuclear breakdown and DNA fragmentation using APO-BrdU TUNEL Assay and /or assessing phosphatidylserine externalization using fluorescent conjugates to annexin V, a phospholipid – binding protein; 4) cell invasion assays which are based on the ability of invasive cells to degrade matrix proteins coating an 8 micron - pore size polycarbonate membrane and their subsequent migration into the lower compartment of the culture chamber; and 5) trans-endothelial migration assays which can be performed in 6.5mm - diameter Transwell Plates. A monolayer of human vascular endothelial cells is grown on gelatin - coated filters (3 micron – pore size for lymphocytic leukemia cell lines and 8 micron –pore size for monocytic leukemia cell lines). A cocktail of chemotactic agents is added to the lower chamber to mediate migration of cells into the lower chamber.

We will further validate the therapeutic efficacy of this peptide (pure and/or KLAKLAK – conjugated) in mouse models of leukemia. The choice of the leukemia model will be based on: 1) the data generated from *in vitro* experiments considering the most sensitive cell line; 2) the efficiency of engraftment of the selected leukemia cell line into SCID or NUDE mice following irradiation; and 3) aggressiveness of the leukemia model. Test and control peptides (350ug per mouse per day) ± chemo will be administered IV, 3 injections weekly for two months. A control group of mice will be treated with the choice of chemo only. Immuno-histochemistry will be done on tissue sections derived from bone marrow, spleen, and liver at various time points during the experiment. Survival of mice will be monitored daily.

**Significance:**
These findings will have important clinical implications in that the newly identified peptide may serve as a peptidomimetic drug lead and can be optimized as a delivery vehicle for targeted therapy of leukemia. This approach has already proved to be fruitful through similar work published recently by our group (Zurita AJ et al., 2004).

**References:**

1. Ravandi F, Talpaz M, Estrov Z. Modulation of cellular signaling pathways: prospects for targeted therapy in hematological malignancies. *Clinical Cancer Research* 9: 535-550, 2003.
2. Giordano R, Cardó-Vila M, Lahdenranta J, Pasqualini R, Arap W. Biopanning and Rapid Analysis of Selective Interactive Ligands. *Nature Medicine* 11: 1249-1253, 2001.
3. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform - specific receptor for vascular endothelial growth factor. *Cell* 92: 735-745, 1998.
4. Latil A, Bieche I, Pesche S, Valeri A, Fournier G, Cussenot O, and Lidereau R. VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. *International Journal of Cancer* 89: 167-171, 2000.
5. Schuch G, Machluf M, Bartsch G Jr., Nomi M, Richard H, Atala A, and Soker S. In vivo administration of vascular endothelial growth factor (VEGF) and its antagonist, soluble neuropilin-1, predicts a role of VEGF in the progression of acute myeloid leukemia in vivo. *Blood* 100: 4622-4628, 2002.
6. Zurita AJ, Troncoso P, Cardó-Vila M, Logothetis CJ, Pasqualini R, Arap W. Combinatorial Screenings in Patients: The Interleukin-11 Receptor $\alpha$ as a Candidate Target in the Progression of Human Prostate Cancer. *Cancer Research* 64: 435-439, 2004.