# Combining Rational and High-Throughput Strategies for the Identification of ALIX-V Ligands of Interest as Broad-Spectrum Antivirals


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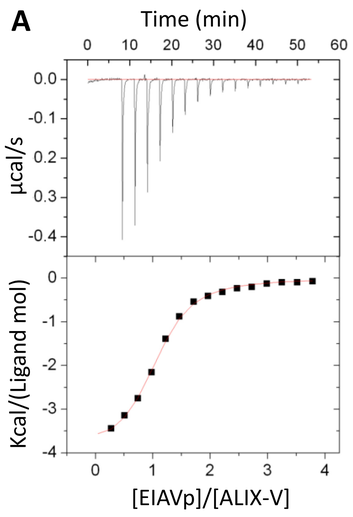
## ABSTRACT

Most membrane-enveloped viruses (including retroviruses and RNA viruses) bud from infected cells by recruiting the host ESCRT machinery through the direct interaction of short and conserved viral sequences called Late domains with some cellular factors. Small-molecule inhibitors of these interactions efficiently block the egress of a number of viruses and are interesting candidates as novel broad-spectrum antivirals. LYP(x)nL Late domains bind to the V domain of the adaptor protein ALIX (ALIX-V), which is a fairly unexplored domain. We present here a thermodynamic study of the molecular determinants of ALIX-V peptide recognition using Isothermal Titration Calorimetry and MicroScale Thermophoresis together with a phage-display analysis of the binding preferences in ALIX-V peptide ligands. Also, the druggability of ALIX-V has been assessed by the high-throughput screening of large compound libraries in search for small-molecule inhibitors of ALIX-V/Late domain interactions. The screening of different compound and drug-repurposing libraries using virtual screening methodologies combined with miniaturized Thermofluor and AlphaScreen® assays has allowed the identification of a set of compounds that bind ALIX-V with IC50 values in the low micromolar range. Bimolecular Complementation Assays validated some of these compounds as ALIX-V/Late domain interactions inhibitors in a cellular context. In order to further explore the druggability of ALIX-V in a richer chemical space we have developed miniaturized HTRF® and AlphaScreen® assays for the screening of the large library of actinomycetes and fungi extracts of the Fundación MEDINA. A pilot assay of a subset of extracts has allowed the identification of one novel compound with IC-50 in the low micromolar range. This work provides new insights of the interaction of ALIX-V with the viral proteins and paves the way towards the development of novel broad-spectrum antivirals.


## 1. The binding of YPx$_{(n)}$L viral Late domains to ALIX-V is weak and entropically driven, suggesting a highly hydrophobic interaction




B

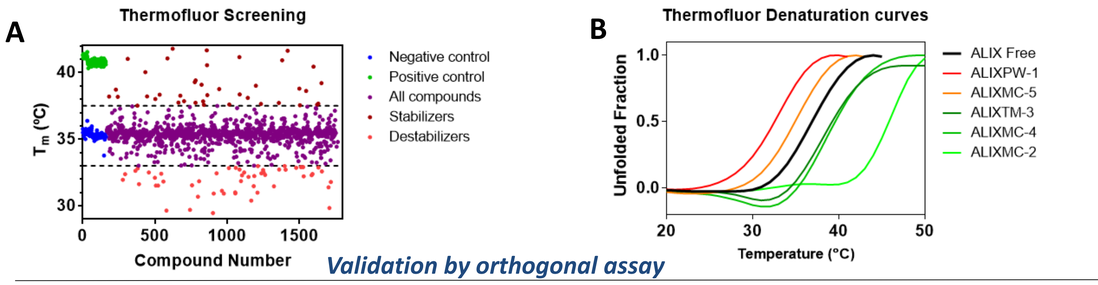
| YPX(n)L Late domain peptides | Sequence | n | Kd (µM) | ΔG (kJ/mol) | ΔH (kJ/mol) | -TΔS (kJ/mol) |
|---|---|---|---|---|---|---|
| EIAV p9Gag | Ac-PQTQNL**YP**D**L**SEIKK-NH2 | 1.2 | 5.1 | -30.2 | -15.1 | -15.1 |
| HIV p6Gag | Ac-IDKEL**YP**LTS**L**RSLFG-NH2 | 0.9 | 3.8 | -30.9 | 6.3 | -37.2 |
| SIV mac239 | Ac-SREK**PY**KEVTED**LL**HLNSLF-NH2 | 1.0 | 10.0 | -28.5 | -13.2 | -15.3 |

**A. Isothermal titration calorimetry**. Upper panel: Titration of the EIAV Late domain into ALIX-V (black line) and dilution experiment (red line). Lower panel: binding isotherm (black squares) and best fit to a one set of sites model (red line).

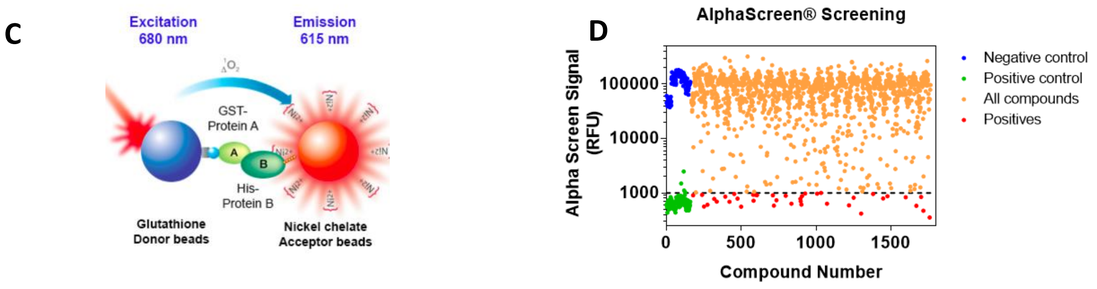
**B. Binding energetics of Late domain peptides to ALIX-V.** YPx$_{(n)}$L viral Late domains bind ALIX-V to a unique binding site with dissociation constants in the low micromolar range and a thermodynamic signature typical of hydrophobic-dominated interactions (small binding enthalpies and favorable entropic contributions).

## 2. ALIX-V and p6-Gag Late domain interactions can be blocked by small molecules

*Primary Screening*




*Validation by orthogonal assay*



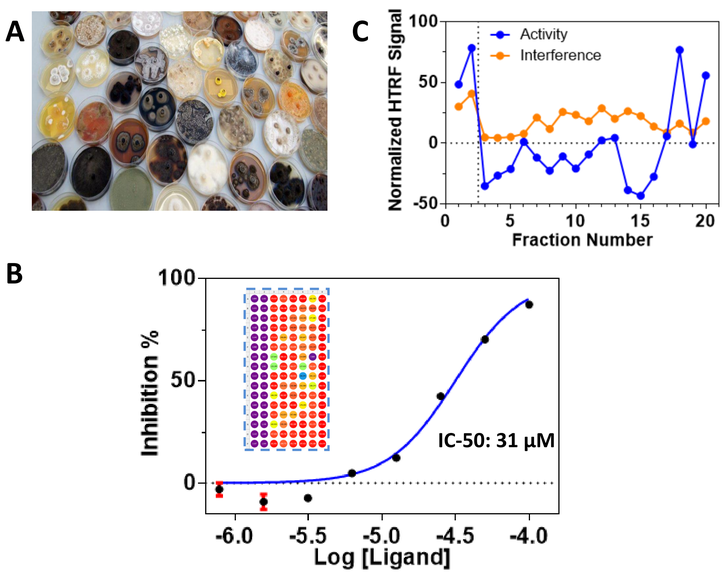

**A. Primary high-throughput screening of drug repurposing libraries using differential scanning fluorimetry (DSC-Thermofluor)** to identify small-molecule binders of ALIX-V. Compounds capable of changing the denaturation temperature of ALIX-V to higher than 37.5º C or lower than 33 ºC were selected for further study.

**B. Normalized DSF profiles for ALIX-V.** Shown are the normalized traces of the free ALIX-V domain (black) and some stabilizing (green) and destabilizing (red/orange) ligands.

**C. AlphaScreen® was used as a secondary orthogonal assay for validation**. The His-tagged ALIX-V protein interacts with a Nickel chelate bead as the GST-tagged p6-Gag protein (HIV) interacts with a Gluthatione bead. The interaction between His-tagged ALIX-V and GST-tagged HIV_p6-Gag protein provides a readable signal. The presence of compounds blocking this interaction leads to loss of luminiscence signal.

**D. Orthogonal high-throughput screening of drug repurposing libraries by AlphaScreen®** to identify small-molecule able to disrupt the ALIX-V/HIV-p6-gag interaction. Compounds that lowered the RFU below 1000 units were selected. A 40% coincidence between the two screening campaigns was obtained.

## 3. A screening of the Fundación MEDINA collection of actinomycetes and fungi extracts has allowed the identification of natural products with the ability to disrupt ALIX-V/HIV_p6-gag interaction.

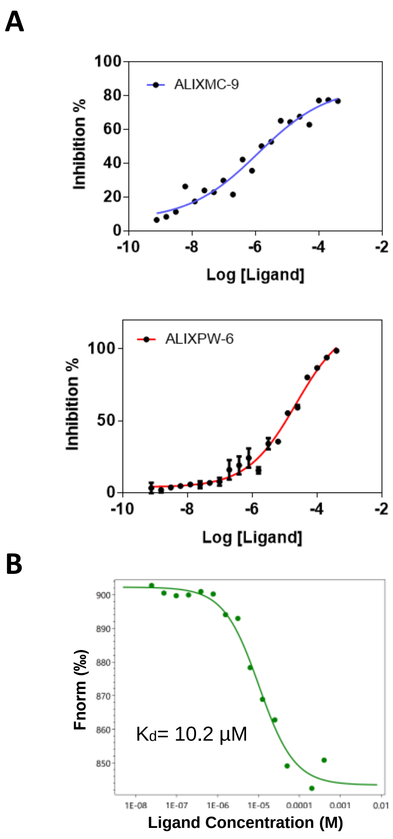



**A. Fundación Medina owns one the world's largest Microbial Collections and Natural Products Libraries** for the discovery of new drugs for unmet medical needs, providing a wide and unexplored chemical diversity.

**B. Primary screen of MEDINA collection of pure natural compounds using Homogeneous Time-Resolved Fluorescence (HTRF®).** HTRF® dose response curve of the MDN-252 compound selected in the primary screen, showing a IC-50 = 31 µM. An example of an assay plate in the primary screen is shown in the inset.

**C. Screening of MEDINA collection of microbial extracts.** Shown is the HTRF® signal for the fractionation profile of an active Fungi extract selected in the HTRF® primary screen. Fractions 1 and 2 correspond to the raw extract. Fractions 3-20 correspond to the increasingly hydrophobic fractions obtained by liquid chromatography of the raw extract. The ability of each fraction to block ALIX-V/HIV-1_p6-gag is shown in blue, and the nonspecific interference calculated from the donor fluorophore emission is shown in orange. The active compound is located in Fraction 18.

## 4. A small set of repurposing drugs can disrupt the ALIX-V/HIV1-p6-gag with IC-50 values in the micromolar range




C

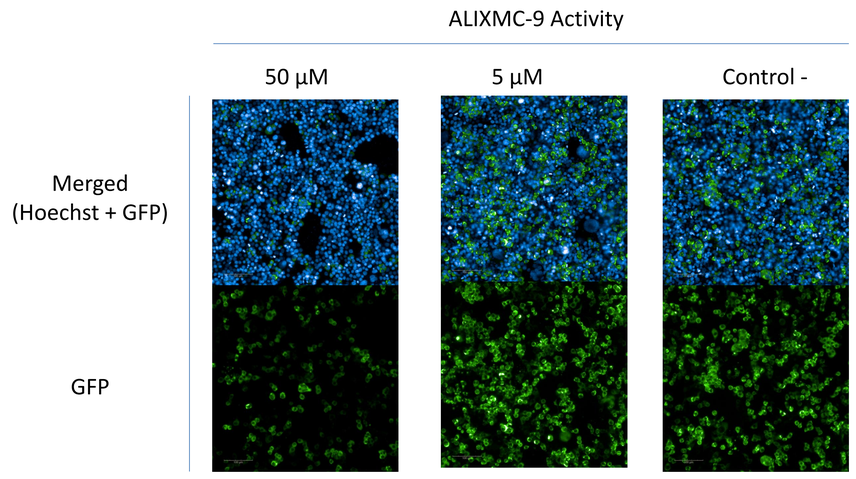
| Name | IC-50 (µM) | Kd (µM) | Indicated treatment |
|---|---|---|---|
| ALIXTM-1 | 3.9 | 10.2 | Cancer |
| ALIXTM-2 | 67.2 | | Others |
| ALIXTM-3 | 50.7 | | Cancer |
| ALIXTM-4 | 117.7 | | Immune system/Digestive system |
| ALIXTM-5 | 0.9 | | Cardiovascular system |
| ALIXMC-3 | 121.9 | 112 | Metabolic Disease; Inflammation |
| ALIXMC-7 | 15.1 | | Cancer |
| **ALIXMC-9** | **1.2** | | Endocrinology |

**A. Validation by AlphaScreen® of selected positives.** Dose-response curves obtained by AlphaScreen® competition assays against a preformed ALIX-V/HIV-p6-gag complex. Symbols represent the average of three independent replicas and error bars show the corresponding standard deviations.

**B. MicroScale Thermophoresis titration of ALIX-V with a drug-repurposing positive**. The experiments were conducted at a 125 nM concentration of ALIX-V with the Monolith His-Tag Labeling Kit RED-tris-NTA. The data was adjusted to a two-state model to determine the binding constants.

**C. The identified repurposing drugs disrupt the ALIX-V/HIV-p6_gag interation with IC50 values in the micromolar range.** Shown are the IC-50 values determined by AlphaScreen® as well as the dissociation constants measured by MicroScale Thermophoresis for some of the ligands.

## 5. Small-molecule inhibitors can block the interacction between the full-length ALIX-V and HIV_p6-gag proteins in a cellular context




**Cellular Images of the Bimolecular Fluorescence Complementation (BiMC) Cellular Assays.** Green Fluorescence Protein (GFP) signal is produced upon interaction between the ALIX and HIV_p6 proteins. Some of the identified ALIX ligands were found to block the interaction between the full-length proteins without significant toxicity. The figure contains the results after ALIXMC-9 treatment at 50 and 5 µM.

## Conclusions

- The thermodynamic characterization show new insights that are key to understand ALIX-V interactions and to approach the design of optimized, high-affinity ligands.
- The ALIX-V binding interface is druggable. Drug repurposing molecules and natural compounds with the ability to block the interaction between ALIX-V and viral late domains in vitro have been identified, which show good potential as novel broad-spectrum antivirals.
- Our screening methodology can readily provide new compounds with the ability to disrupt the host-virus proteins interaction in a cellular context, validating the target for antiviral discovery.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