Reaching the target – a novel cell penetration assay provides new insights into the design and intracellular compartmentalization of biotherapeutics

Review Article

There have been massive advances in the development of biotherapeutics but progress has been slow in targeting intracellular processes due in part to the difficulty of measuring cell penetration and distribution between intracellular compartments and organelles. A research group based at Tufts University, USA, has developed a novel assay, the Chloroalkane Penetration Assay (CAPA), as a powerful tool to meet this challenge. They have shown how CAPA can help in following the modulation of biomolecule cell penetration, including structure-penetration profiling of bioactive stapled peptides and peptides fused to cell-penetrating sequences, measuring the effect of environmental factors on penetration, and tracing the movement of potential biotherapeutics between organelles.

Cell penetration – the challenge of ensuring biotherapeutics reach their intracellular target

The high affinity and specificity of biomolecules such as peptides, antibodies, and nucleic acids makes them promising biotherapeutics with excellent potency and minimal off-target effects. Biotherapeutics are dominated by monoclonal antibodies that target extracellular receptors. Smaller biomolecules such as peptides can be modified to improve cell penetration, for example, by modifying helical peptides with all-hydrocarbon staples. Improving the cell penetration of larger biomolecules, however, has proved to be challenging.

Pioneering work provides cell penetration tools

In 1988, two research groups were the first to observe a protein, the human immunodeficiency virus 1 (HIV 1) trans-activating (Tat) protein, crossing the cell membrane of tissue-cultured cells, and translocating into the nucleus to transactivate viral gene expression. The α-helical domain of the Tat protein, mainly composed of basic amino acids, proved to be the primary determinant for this cell internalization and nucleus translocation (see, for example, reference 1).

The Tat dodecapeptide GRKKRRQRRRPQ has since been shown to be the minimal functional molecule required to promote cell penetration. As a result, this peptide has formed the basis for many polycationic cell-penetrating peptides (CPPs) that are conjugated to biomolecules as a common method to promote cell penetration. Since then, a considerable number of CPPs have been investigated for the treatment of several diseases, including infections, inflammation, neurodegenerative disorders and cancer.

The chloroalkane penetration assay (CAPA) enables cell penetration profiling

One of the greatest challenges in understanding and improving cell penetration is determining localization in the cytosol. Many methods involve tagging molecules with a fluorescent dye and tracking them using microscopy. But these methods cannot resolve localization to cytosolic and endosomal compartments and, while alternative

methods address some of these drawbacks, there is still a need for high-throughput assays that can deliver compartment-specific, quantitative data for cell-penetrating biomolecules.

A research group based at the Department of Chemistry, Tufts University, Massachusetts, USA, has developed a new assay for measuring cell penetration called the chloroalkane penetration assay (CAPA; 2). This assay uses cells stably expressing HaloTag (Promega), which is a modified form of bacterial haloalkane dehalogenase that rapidly labels itself covalently with the chloroalkane. The pulse-chase assay starts with a compound labeled with a chloroalkane tag (ct; Figure 1). If this compound penetrates the cell and enters the cytoplasm then it will covalently block the HaloTag. A chase with a chloroalkane dye will enable the quantification of the HaloTag that was not labeled by the test molecule. Measurements are made by flow cytometry and data is commonly expressed as CP_{50}, the concentration at which 50% cell penetration is observed.

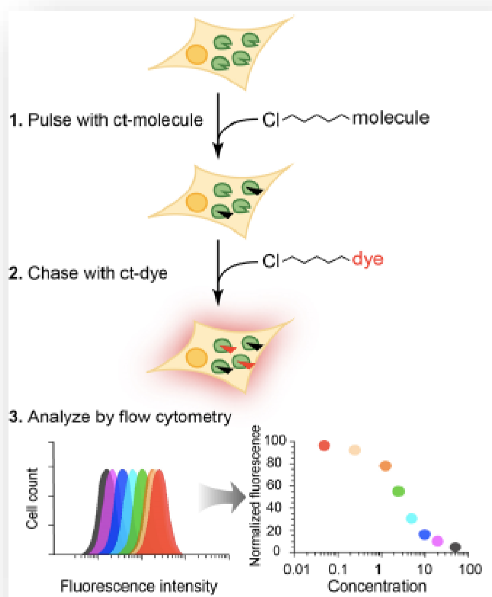

Figure 1. *The set up of a CAPA. Schematic showing assay steps. Cells stably expressing HaloTag are pulsed with chloroalkane-tagged molecules, washed, chased with a chloroalkane-tagged dye, and finally analyzed by flow cytometry. From Figure 1a, Peraro et al, 2018.*

This assay has enabled the research group to generate considerable amounts of data to characterize cell penetration by bioactive stapled peptides and peptides fused to cell-penetrating sequences over a large range of concentrations, time scales, and culture conditions, including structure-penetration studies.

Peptide synthesis and chloroalkane tagging

Peptides were synthesized on Rink Amide resin using standard Fmoc chemistry, by hand or using a Tribute® automated peptide synthesizer. Each coupling involved dissolving benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIPEA) in N,N-dimethylformamide (DMF), which was added and shaken with the resin for 30 min at rt. Deprotection was done with a piperidine/DMF solution. A final N-terminal capping with the chloroalkane carboxylate was done using PyBOP and DIPEA in DMF. The coupling efficiency was checked and a second coupling was performed if reaction was incomplete.

Peptides were cleaved from the resin using TFA/EDT/H_2O/TiPS and precipitated in diethyl ether. Peptide ct-DD5o was cyclized using thiol bisalkylation chemistry. The linear peptide DP1 (FΦRRRREKW) was synthesized with Fmoc-Glu-OAll and Fmoc-Lys(Mtt)-OH, and cyclized by deprotecting the Glu, washing, deprotecting the N-terminal Fmoc, and cyclizing using PyBOP, HOBt, and DIPEA in DMF. The Mtt group on the Lys was deprotected and then reacted with the chloroalkane carboxylate. Peptides were purified by RP-HPLC and lyophilized.

Peptides were tagged with chloroalkane using a chloroalkane carboxylate and peptides tested in this work were generally conjugated to the chloroalkane carboxylic acid on their N-terminus.

Table 1: Sequences

Amino acid/Peptide	Sequence	Staple
ct-W	ct-W	–
ct-Tat	ct-YGRKKRRQRRR	–
ct-Arg9	ct-RRRRRRRRRW	–
ct-SAHB	ct-IWIAQELR(*S5*)IGD(*S5*)FNAYYARR	hydrocarbon
ct-DD50	ct-VcNAcFHIWH	*o*-xylene

These are the sequenced mentioned in this summary. ct, chloroalkane tag. From SI Table 1, Peraro et al, 2018.

Cell penetration profiling

CAPA can be adapted to quantify cell penetration under different incubation conditions. For example, in an experiment that involved a panel of molecules spanning different classes of cell-penetrant molecules, increasing the temperature greatly increased the cell penetration of ct-SAHB, ct-DD50, and ct-W, while ct-Tat and ct-Arg9 were less affected (Figure 2).

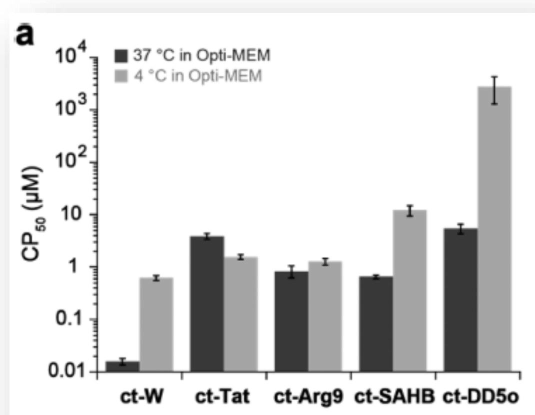

Figure 2. *Profiling of cell penetrants. The effect of temperature on the cell penetration of the small molecule W, two widely used polycationic CPPs, Tat and Arg9, and two different classes of stapled peptides, BIM-SAHB$_{A1}$ and DD50. From Figure 2a, Peraro et al, 2018.*

Investigating the structure-penetration relationship

CAPA can also be used to optimize the type and location of CPPs for CPP-conjugated peptides. Earlier work using an alanine scan of peptide DD50 had yielded structure-activity relationships for this stapled, autophagy-inducing peptide. Induction of autophagy is normally judged by phenotypic methods but these cannot differentiate the effects of cell penetration from other properties. The researchers therefore used CAPA to investigate structure-penetration relationships more directly. For example, substitution of Val1, Asn3, or Phe6 to Ala had marginal effects on penetration, whereas substituting His7 or His10 with Ala increased the CP_{50} four-fold, and substitution of Trp9 to Ala essentially abolished cell penetration (Figure 3).

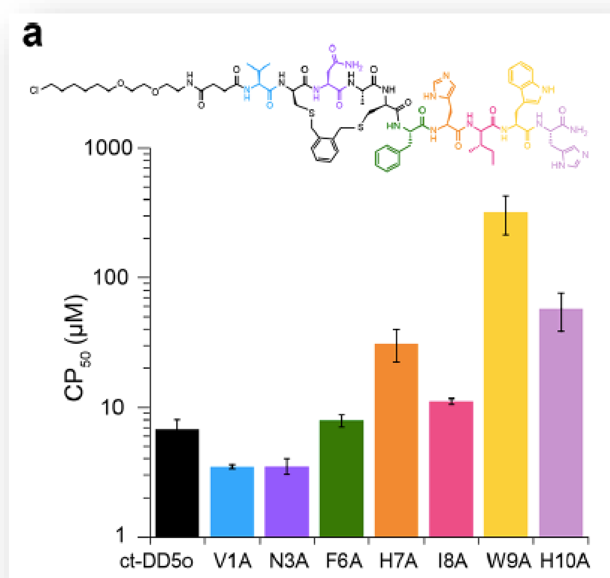

Figure 3. *Structure-penetration relationships. CAPA results for alanine-scanning analogues of ct-DD50. Note that the CP_{50} values are shown in a log scale. From Figure 4a, Peraro et al, 2018.*

A powerful tool in biotherapeutic development

CAPA has a wide register of applications. In addition to the profiling and structure-penetration studies, the research group also demonstrated the ability of CAPA to measure cell penetration over time with a resolution of 1–2 hours. Added to that, they could follow the penetration of molecules into the nucleus, which underlines the value of CAPA in the design of drugs targeted to specific subcellular compartments or organelles. The method can also be used to follow the cell penetration of nucleic acids and proteins. This quantitative, high-throughput and compartment-specific method therefore shows great promise in medicinal chemistry and compound optimization.

For a more detailed description of Dr. Kritzer's work using the CAPA assay, please listen to the webinar.

References

1. Cell penetrating peptides as molecular carriers for anti-cancer agents. Borrelli A et al. Molecules. 2018 Jan 31;23(2). pii: E295. doi: 10.3390/molecules23020295.
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