In Vitro Membrane Affinity Measurements may increase the Predictability of Calculated Permeability Properties of Macrocycles

Poster P2

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ABSTRACT

PURPOSE:

Macrocyclic peptides (MCPs) are gaining interest as potential therapeutics due to their notable target selectivity, binding affinity, and ability to disrupt protein-protein interactions. Permeability across plasma membranes is a major hurdle for MCPs and can limit access to intracellular targets and negatively impact oral absorption. Understanding how complex 3-dimensional conformations of MCPs interact with phospholipids and plasma membranes on a fundamental level is needed to design permeable MCPs.

METHODS:

We optimized and automated the TRANSIL membrane affinity kit (Sovicell) on a Hamilton VANTAGE to assess the membrane affinity (Log MA) of MCPs. Four MCPs per cassette (0.5 µM/MCP) were added to wells containing phospholipids (PLs) surrounding silica beads. A constant concentration of MCP was added to wells with varying concentrations of PLs. Equilibrium between MCPs and PLs was reached by mixing the beads with the 96-channel head for 120 cycles (200 µL/mix, 250 µL/s). Supernatant was collected after centrifugation and analyzed on a Sciex ZenoTOF 7600. The phospholipid composition used in this study approximates that of MDCK cells; 50% phosphatidylcholine (PC), 17%, phosphatidylethanolamine (PE), 11% phosphatidylserine (PS), 2% phosphatidylinositol (PI), 20% other PLs. The Log MA was calculated using the algorithm supplied with the kit. Additionally, we ran the Schrodinger Prime Macrocycle Permeability calculation, which estimates the free energy for an MCP to move from water into a membrane (dG_insert). dG_insert has been shown to correlate with passive permeability for both small molecules and small macrocycles. Since bigger MCPs pose challenges due to the large number of rotatable bonds and thus the size of the conformational space, dG_insert was calculated for MCPs with MWs < 1000.

RESULTS:

As expected, Log MA is not predictive of apparent permeability obtained from our 96-well MDCK permeability assay (R^2: < 0.1, n=219 MCPs). While dG_insert was highly correlated with EPSA (R^2 = 0.82, n=74), it was not with MDCK Papp (R^2: 0.36, n=85). The correlation between Log MA and dG_insert (R^2: 0.36, n=113) was minimal. Out of 85 MCPs with MDCK Papp, Log MA and dG_insert values, 16 had MDCK Papps >= 0.5 10E-06 cm/s. A dG_insert value < 9 predicted 24 MCPs would have acceptable permeability (8 false positives). When dG_insert (< 9) and Log MA (2.5 – 4.5) were used together as cut-offs, 14 out of 16 MCPs were predicted to have acceptable permeability (87.5%).

CONCLUSIONS:

Therefore, the Log MA from the Transil membrane affinity assay may increase predictability when combined with calculated properties over either alone.

INTRODUCTION

A disconnect between PAMPA permeability and permeability in gMDCK cells was observed for most of the 40 MCPs tested. The permeability in PAMPA was markedly higher when compared to gMDCK. Since the gMDCK cell line has endogenous canine Mdr1 gene knocked out, efflux by this transporter is not the cause of the disconnect. Some possible reasons for the lack or correlation between the permeability assays explored in this work for a subset of 11 MCPs include length of incubation (PAMPA, 16h; gMDCK, 3 h and 24 h), poor metabolic stability of the MCPs in the cells, lysosomal sequestration of the MCPs as they traverse the cells, and that PAMPA and MDCK cells have significantly different phospholipid membrane compositions (Table 1).

Table 1: Cell, PAMPA, and Transil Membrane Phospholipid Composition

Phospholipid	Typical Mammalian Cell[1]	Transil MDCK PL Mix	Transil PAMPA PL Mix
Phosphatidylcholine (PC)	52	50	95
Phosphatidylethanolamine (PE)	22	17	0
Phosphatidylserine (PS)	12	11	0
Phosphatidylinositol (PI)	14	2	0
Other/uncharacterized PLs	0	20	5
[1]Treyer et al. Pharm Res (2019) 36: 178		TMP-0170-2096	TMP-0160-2096

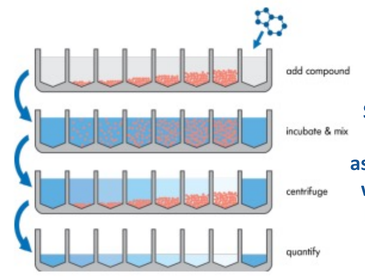


Figure 2
Schematic of Transil membrane binding assay procedure which was automated on a Hamilton VANTAGE

MATERAILS and METHODS

MCPs: The 11 MCPs tested across all experiments had molecular weights ranging from 1060 – 1186, cLogP values of 3.5 – 5.7, and TSPA values of 256 – 301.

PAMPA Experiments:
PAMPA membranes were made by dissolving egg lecithin (1.8% w/v) in N-dodecane and adding 5 µL on to a well of a 96-well plate (PVDF membrane). MCPs (50 µM) were incubated for 16 hours at 37C after which samples were collected and injected onto a LC/MS/MS.

96-Transwell MDCK Permeability Experiments:
Cell Culture: gMDCK cells (Mdr1 KO) were seeded at 75,000 cells/well on Corning 96-Transwell plates with PET inserts (1.0 µm) and grown to confluence (37°C, 5% CO2, 95% humidity) for 48 hours. Cells were used in experiments approximately 48 – 55 hours post-seeding.

Standard 3h Experiment: MCPs were diluted in assay buffer (Hank's balanced salt solution) containing 10 mM HEPES buffer and 50 µM lucifer yellow, pH 7.4. Cells were washed 3 times with assay buffer before MCPs were added (10 µM, final DMSO concentration 1%). After 3 hours of incubation samples were collected.
24 h Preincubation Experiment: After 48 h hours in culture, the cell culture medium was removed. Fresh DMEM media containing 10% FBS and 10 µM of MCPs was added to both the apical and basolateral chambers. Cells were incubated in the presence of MCPs for 24 hours. On the day of the experiment, media containing MCPs was removed and cells were rapidly washed 3 times with assay buffer. MCPs in assay buffer and lucifer yellow were added to the cells and the experiment was run using the 3 h protocol.

gMDCK Cell Lysate Stability Experiment: gMDCK cells were grown to confluence in T175 flasks. On the day of the experiment, flasks were trypsinized for 10-15 minutes. Cells in suspension were washed 3 times in PBS, diluted to 1 million cells mL in permeability assay buffer and sonicated (two pulses at 20 seconds/pulse) to lyse the cells. MCPs (1 µM) were added to lysates in the presence and absence of protease inhibitor cocktail. MCPs were incubated at 37°C with shaking (100 RPM). Time points were collected at time 0, 5, 15, 30, 60, and 120 minutes and the reaction was quenched using 100% acetonitrile containing analytical internal standard and 5% formic acid. Samples were vortexed, centrifuged and diluted 3:1 with mobile phase prior to analysis.

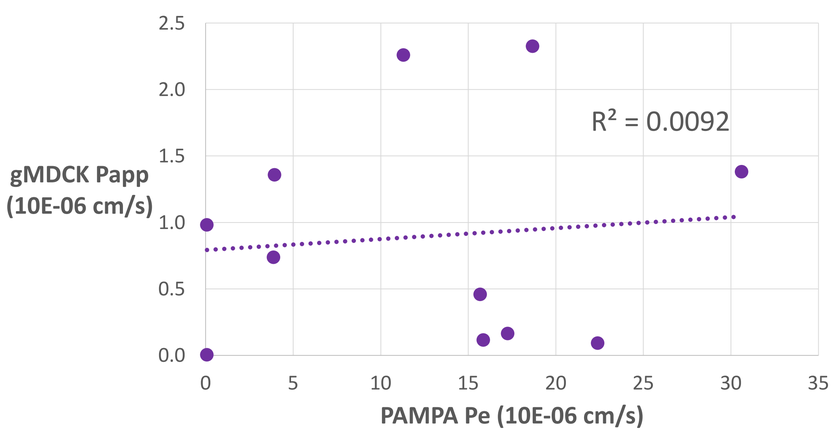
Lysosomal Trapping Experiment: Bafilomycin A (BafA) inhibits the V-type ATPase in the lysosomal membrane responsible for maintaining the lysosome's acidic pH (5.5). A modified 3 h gMDCK permeability protocol was used to determine if MCPs were sequestered in cells during a permeability experiment. The first group (lysosomal trapping group), the experiment was run using the 3 h protocol. Group 2, included a 45 minute preincubation with BafA (1 µM). Additionally, BafA (1 µM) was included in the experiment with MCP (10 µM) and in the receiver chamber assay buffer.

Sovicell Transil Membrane Affinity Measurements: Transil PC and intracellular binding membrane affinity kits (part numbers TMP-0160-2096 and TMP-0170-2096, respectively) were thawed overnight at 4°C. On the day of the experiment, the kits were equilibrated at room temperature for approximately 1 – 1.5 h before starting the experiment. The kits were centrifuged for 10 seconds at 750 x g (20°C). The Hamilton VANTAGE added 15 µL of a 8 µM MCP stock to the beads to achieve a final concentration of 0.5 µM for each of the MCPs in the cassette. The plates were mixed using the 96-channel pipetting head for 120 cycles (200 µL/mix, 250 µL/s. Figure 1). The beads were centrifuged at 750 x g for 10 minutes (20°C) and the 96-channel pipette head was used to transfer 50 µL of the supernatant to an analysis plate preloaded with 100 µL of internal standard (50% ACN:50% water, 1% formic acid). Log membrane affinity values (Log MA) were calculated using a modified version of the calculation template provided with the kits.

Analytical: gMDCK permeability and lysates samples, Transil membrane affinity samples and samples from the lysosomal sequestration experiment were analyzed on a ZenoTOF7600 mass spectrometer.

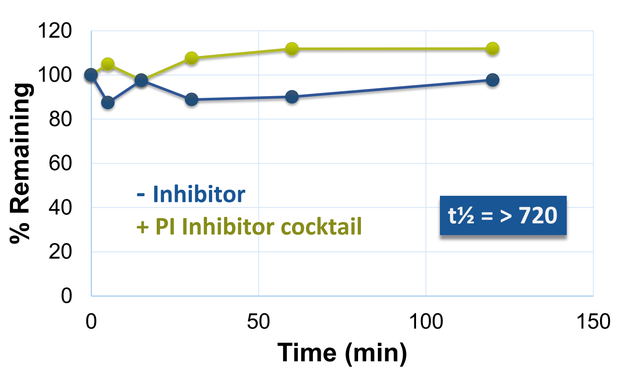
RESULTS

Figure 2
PAMPA 16h vs 96-TW gMDCK 3h



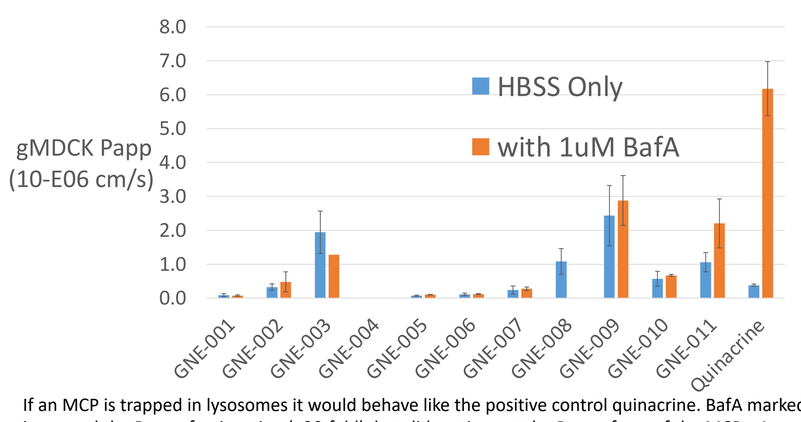
Poor correlation between PAMPA and 3 h gMDCK permeability experiments.

Figure 3
Representative Stability of MCPs (GNE-005) in gMDCK Cell Lysate



All 11 MCPs were stable in the presence and absence of protease inhibitor (PI) cocktail and had t½ values > 720 minutes.

Figure 4
Lysosomal Trapping of MCPs in gMDCK Cells



If an MCP is trapped in lysosomes it would behave like the positive control quinacrine. BafA markedly increased the Papp of quinacrine (~30-fold), but did not impact the Papp of any of the MCPs. A secondary measure of lysosomal trapping is mass balance. BafA increases the mass balance of trapped compounds. The mass balance of the MCPs was not impacted (data not shown), therefore it appears the MCPs are not trapped in lysosomes.

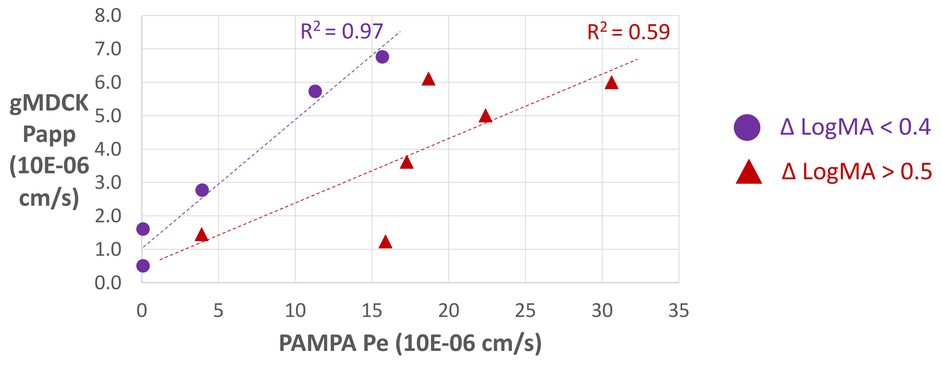
Figure 5
Log MA, PAMPA Pe and gMDCK 3 h and 24 h Experiment Papp Values

	Transil Log MA		PAMPA Pe (10E-06 cm/s)	gMDCK Papp (10E-06 cm/s)	
	PAMPA PL Mix	MDCK PL Mix	16 h Incubation	3 h Incubation	24 h Preincubation
GNE-001	**2.0**	**2.5**	22.4	0.09	5.01
GNE-002	2.6	2.8	15.7	0.46	6.76
GNE-003	3.3	3.4	0.08	0.98	1.60
GNE-004	2.0	1.6	0.08	0.003	0.50
GNE-005	**0.7**	**1.8**	15.9	0.11	1.24
GNE-006	**1.3**	**2.0**	17.3	0.16	3.62
GNE-007	2.9	3.0	11.3	2.26	5.73
GNE-008	3.0	3.2	3.9	1.36	2.76
GNE-009	**2.7**	**3.9**	18.7	2.32	6.11
GNE-010	**3.5**	**4.5**	3.9	0.74	1.45
GNE-011	**2.0**	**2.9**	30.6	1.38	6.01

BOLD = difference between PAMPA and MDCK PL mix Log MA values >= 0.5

6/11 (55%) of the MCPs tested in the Transil membrane affinity assays had differences in Log MA >= 0.5 between the PAMPA PL mix and MDCK PL mix.

Figure 6
PAMPA 16h vs 96-TW gMDCK 24 h Preincubation with Transil Δ Log MA



gMDCK 24 h preincubation Papp values correlate better with PAMPA than the gMDCK 3 h Papp values. Δ Log MA is the difference between the Log MA from the MDCK PL mix and the Log MA from the PAMA PL mix. In cases where the Δ Log MA is < 0.4, the PAMPA Pe and gMDCK Papp values differ by < 2.5-fold (purple circles). For MCPs where the Δ Log MA is ~ 0.5 – 1.1, the PAMPA Pe and gMDCK Papp values differ by 2.5 to 13-fold (red triangles).

CONCLUSIONS and FUTURE DIRECTION

- All 11 MCPs are stable in cell lysates, therefore metabolism is not the cause of the PAMPA-gMDCK permeability disconnect.
- The MCPs tested are not appreciably sequestered in lysosomes.
- Incubation time of the gMDCK assay seems to play a role in the PAMPA-gMDCK disconnect. One possible reason could be due to the differences in phospholipid composition between the two systems.
- For this set of MCPs, a Δ Log MA > 0.5 between the PAMPA PL mix and gMDCK PL mix results in large differences in PAMPA Pe and gMDCK Papp (2.5 to 13-fold).
- Further investigation using Transil beads containing phosphatidylcholine (20%) and phosphatidylserine (80%) and phosphatidylcholine (20%) and phosphatidylethanolamine (80%) to determine the impact phosphatidylserine and phosphatidylethanolamine may have on Log MA and ultimately the permeability of these MCPs.
- A previously reported $f_{u,cell}$ method for small molecules [Treyer et al. Pharm Res (2019) 36: 178] has been automated for MCPs and beyond rule of five molecules. Validation is currently ongoing.