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Assessing Intestinal and CNS Permeability with MDR1-MDCKII Cells

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Anzhela Rodnichenko[1], Yurii Kheilik[1]

[1]Enamine Ltd

ASAP Discovery

Nick Lynch
Curlew Research, ASAP Discovery Consortium

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We use this protocol and it's working

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Disclaimer

Abstract

The protocol allows measurement of the rate of flux of a compound to identify intestinal or CNS permeability. The assay measures the rate of flux across Canine MDR1 Knockout, Human MDR1 Knockin MDCKII cells (MDR1-MDCKII) cell monolayers to identify intestinal or CNS permeability.

By measuring how quickly a compound passes through MDR1-MDCKII cell monolayers, researchers can estimate its intestinal permeability and predict its potential to reach the brain.

Interpreting Results:

- High Permeability: A compound that readily crosses the MDR1-MDCKII monolayer is likely to be well-absorbed from the intestine and may have a high potential to reach the brain.
- Low Permeability: A compound that does not readily cross the monolayer may have limited intestinal absorption or be actively pumped out of the brain by the MDR1 protein.

Guidelines

Introduction:

MDR1-MDCKII cells are often used instead of Caco-2 cells and tend to require a relatively short culture period (3–5 days) while displaying interpassage homogeneity and a high level of human P-gp expression. MDR1-MDCKII cells stably transfer and express the MDR1 gene (ABCB1) in MDCK cells, which encodes and expresses the efflux protein P-gp that causes multiple drug resistance. This protocol describes the permeability in the bidirectional permeability assay.

CALCULATION OF PERMEABILITY COEFFICIENT:

The apparent permeability coefficient (Papp, unit: cm s^{-1}) is determined from the amount of compound transported per time; Papp is usually calculated according to the following equation:

Papp is expressed in 10^{-6}cm/sec.

$$P_{app} = \frac{V_A}{Area \times Time} \times \frac{[drug]_{acc}}{[drug]_{initial,d}}$$

VA – volume of transport buffer in acceptor well,
Area – surface area of the insert (0.7 sq.cm)
Time – time of the assay,
[drug]acc – peak area of the test compound in acceptor well,
[drug]initial,d – initial peak area of the test compound in a donor well.

CALCULATION OF EFFLUX RATION:

Usually, perform transport experiments in both directions across the cell monolayers. The ratio between the two permeability coefficients obtained can then be used as a first indication of the involvement of an active transport process. If there is no clear difference between the permeability coefficients in the two directions, the transport might be passive. The Efflux ratio reveals the difference in Papp as a result of active transport.

Efflux ratio is calculated according to the following equation:

$$P_{app}(BA)/P_{app}(AB)$$

Note

The intestinal barrier is composed of multiple parallel transport processes including passive diffusion (transcellular and paracellular) and active diffusion (carrier-mediated absorption and carrier-mediated efflux). If the efflux ratio is less than 2, this indicates that the compound is passively transported. If the efflux ratio is greater than 2, this indicates the occurred active efflux.

CALCULATION OF MASS BALANCE:

The % recovery (mass balance) can be useful in interpreting the MDR1-MDCKII data. If the recovery is very low, this may indicate poor solubility, binding of the compound to the test plate materials, metabolism by the MDR1-MDCKII cells, or accumulation of the compound in the cell monolayer. The % recovery was calculated using the following equation:

$$\%\,\boldsymbol{recovery} = \frac{\boldsymbol{C_{acc}} \times \boldsymbol{V_{acc}} + \boldsymbol{C_d} \times \boldsymbol{V_d}}{\boldsymbol{C_{initial,d}} \times \boldsymbol{V_d}} \times \mathbf{100}$$

Vacc – volume of compound solution in acceptor well (cm^3),
Vd – volume of compound solution in donor well (cm^3),
Cacc – peak area of test compound in acceptor well,
Cinitial,d – initial peak area of test compound in a donor well.

Note

The recovery should be kept within limited ranges in order to assure high-quality and accurate Papp values. Limits for recovery are between 80 and 120%.

Materials

Reagents and equipment:

1. CelCulture® Incubator, CO2 **ESCO lifesciences Catalog #**CCL-170B-8
2. Centrifuge 5804R (Eppendorf, USA)
3. Etched Hemacytometer, dark line counting chamber (Hausser Scientific, USA; Cat# 3500)
4. Innova 4080 Incubator Shaker (New Brunswick Scientific, USA)
5. Millicell-ERS system ohm meter (Millipore, Cat# MERS 000 01)
6. Gradient HPLC system Prominence UFLC XR (Shimadzu, Japan)
7. VWR Membrane Nitrogen Generators N2-04-L1466, nitrogen purity 99%+ (VWR, USA)
8. Channel Manual Pipette **pipette.com Catalog #**FA16-50R
9. Multichannel Electronic Pipettes 2-125 µL, 5-250 µL, 15-1250 µL, Matrix (Thermo Scientific, USA)
10. PIPETMAN pipettes 2-20 µl, 50-200 µl, 200-1000 µl (Gilson, USA)
11. Water purification system Millipore Milli-Q Gradient A10 (Millipore, France)
12. Opentrons OT-2 (Opentrons Labworks, USA)
13. Triple quadrupole mass-detector API 3000 with TurboIonSpray Ion Source (AB Sciex, Canada) or Hybrid triple quadrupole/linear ion trap mass-detector API 4000 QTRAP with Turbo V ion source
14. DMEM/HIGH with L-glutamine **HyClone Catalog #**SH30003.04
15. Fetal Bovine Serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F7524
16. Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S5761
17. Penicillin-Streptomycin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P4333
18. Trypsin-EDTA solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T4174
19. HEPES **Santa Cruz Catalog #**SC-29097
20. DPBS, powder, no calcium, no magnesium **Thermo Fisher Catalog #**21600044
21. Hanks' Balanced Salts **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H4891
22. Millicell® 24 well Plate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**PSRP010R5
23. 24 well Collection Tray **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**PSMW010R5)
24. UltraCruz® Centrifuge Tube, Conical, 50 ml, bulk **Santa Cruz Catalog #**sc-200251
25. Serological Pipettes 5 ml, 10 ml, 25 ml (Greiner Bio-One)
26. Disposable pipettor tips (Thermo Scientific, Fisherbrand, Eppendorf USA)
27. Matrix™ Blank and Alphanumeric Storage Tubes **Thermo Fisher Scientific Catalog #**4140
28. Ketoprofen **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**PHR1375
29. Atenolol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**74827
30. Quinidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**Q3625
31. Dimethyl sulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34869

32. Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34851
33. Formic Acid (FA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**94318
34. Methanol suitable for HPLC ≥99.9% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860
35. Heptafluorobutyric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**52411
36. InfinityLab Poroshell 120, EC-C18, 2.1 × 50 mm **Agilent Technologies Catalog #**699770-902 or Phenomenex Luna® C18 HPLC column, 2.1×50 mm, 5 µm (Cat# 5291-126) or ZORBAX RR HILIC Plus Column, 2.1 × 50 mm, 3.5 µm **Agilent Technologies Catalog #**959743-901 or Pursuit XRs 100Å C18, 2.0 x 50 mm, 5 µm **Agilent Technologies Catalog #**A6000050X020

Reagent Preparation:

- **Complete medium:** DMEM high glucose (4500 mg/l) with L-glutamine 4 mM (584 mg/l) supplemented by 10% heat-innactivated FBS, 1% of non-essential amino acids, 10 mM HEPES buffer and 1% Penicillin/Streptomycin solution.

- **Trypsin/EDTA solution prepare** from 10x stock solution by addition of DPBS.

- **Transport buffer preparation:**

Hanks' BSS (1x) without Ca & Mg, without Phenol Red, with $NaHCO_3$ (final concentration 0.35 g liter) supplemented by $MgSO_4$ (final concentration 0.81 mM), $CaCl_2$ (final concentration 1.26 mM), HEPES (final concentration 25 mM).

- **1M stocks solution:**

1. $MgSO_4$ - Dissolve 120.37 g of $MgSO_4$ (anhydrous) or 246 g of $MgSO_4$x $7H_2O$ to 1 liter of distilled H_2O.
2. $CaCl_2$ – Dissolve 110.98 g of $CaCl_2$ or 147,01 g of $CaCl_2$x $2H_2O$ to 1 liter of distilled H_2O.
3. HEPES - Dissolve 238.30 g of HEPES in 1 liter of distilled H_2O.
4. For preparation of 5 L Transport buffer add: 4.05 ml 1 M $MgSO_4$, 6.3 ml 1M $CaCl_2$, and 125 ml 1M HEPES. Adjust the pH to 7.4 and then filter sterile the solution using a 0.22 filter.

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

CULTIVATION OF THE MDR1-MDCKII CELLS BEFORE SEEDING ON THE FILTER 15m

1 Trypsinize MDR1-MDCKII cells from maximally (90%) confluent MDR1-MDCKII cultures: remove the medium, rinse with DPBS (10 mL per 75 cm^2 flask) 2-3 times, decant DPBS and add the trypsin/EDTA solution (3 mL per 75 cm^2 flask); the remaining trypsin must wet the entire cell layer.

1.1 Incubate the flask at for 00:04:00 – 00:10:00 (use the shortest time possible) and check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm. 14m

- As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in a complete medium.

2 Culture MDR1-MDCKII cells in complete medium in 75 cm^2 flasks to 80-90% confluence according to the humidified atmosphere at 37 °C and 5% CO_2.

3 Spin down the cells (1000 rpm, 00:05:00) and remove the supernatant. 5m

4 Take an aliquot and count the cells.

5 Resuspend in the complete medium and seeded at a density 6-7×10^3 cells/cm^2.

Note

After thawing a vial of MDR1-MDCKII cells from the stock, cultivate the cells for two passages before seeding cells on the filter support to stabilize the cell phenotype.

CULTIVATION OF THE MDR1-MDCKII CELLS ON FILTER SUPPORTS 6h 15m

6

Note

Used MDR1-MDCKII cells of passage number 10-20.

Trypsinize MDR1-MDCKII cells from maximally (90%) confluent MDR1-MDCKII cultures: remove the medium, rinse with DPBS (10 mL per 75 cm^2 flask) 2-3 times, decant DPBS and add the trypsin/EDTA solution (3 mL per 75 cm^2 flask); the remaining trypsin must wet the entire cell layer..

6.1 Incubate the flask at 37 °C for 00:04:00 – 00:10:00 (use the shortest time possible) and check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm. 10m

- As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in a complete medium.

7 Transfer the cells to a test tube and allow the large cell aggregates (if any, use 70-µm cell strainer) to sediment. Transfer the supernatant to a new test tube, take an aliquot, and count the cells.

Note

The percentage of dead cells must not exceed 5%.

8 Spin down the cells (1000 rpm, 00:05:00) and remove the supernatant. 5m

9 Resuspend the cells in complete medium (at a concentration of 400×10^3 cells/ml).

10 Seed by dispensing 0.4 mL of the resuspended cell solution on each filter and 25 mL of prewarmed complete medium add to the feeder tray.

Note

Filters with a pore diameter of 3 µm are not recommended, as they allow the cells to crawl through the pores to the opposite side of the filter, resulting in a double monolayer.

11 Incubate the plate with the filter supports at 37 °C and 5% CO_2 in a humid atmosphere for 06:00:00 (if seeding is done in the morning) or Overnight (16 h; if seeding is done at the end of the day). 6h

12 Remove and replace the medium.

Note

This step is done to remove non-adherent cells and to reduce the risk of multilayer formation.

13 Maintain cells, every second day: aspirate the medium from the basolateral side and then carefully and slowly from the apical side (of all filters in a plate). Replace the aspirate medium with fresh, first in the apical compartments and then in the basolateral compartments.

Note

- Do not touch the filter surface with the pipettes! Slow pipetting and avoiding physical contact between the pipette tip and the monolayer are essential for the maintenance of monolayer integrity.
- Permeability testing performed 4 days post seeding.

DRUG TRANSPORT EXPERIMENT 1d 3h 10m

14 **Critical step:** Change the culture medium 12:00:00 – 24:00:00 before the experiment. 1d

Note

Longer periods without feeding before starting the experiment should be avoided because the cells may have consumed the essential nutrients and adapted to a more starved phenotype.

15 Equilibration plates from 37 °C to Room temperature at 00:20:00 - 00:30:00 . 30m

Note

- TEER measurements are temperature dependent. Ideally, TEER measurements should be conducted in an incubator at 37 °C .
- If TEER measurements are performed at Room temperature , it would be necessary to first equilibrate temperature before performing TEER measurements to avoid any temperature fluctuation-induced TEER changes.
- Typically, equilibration from 37 °C to Room temperature requires at least 00:20:00 .

16 Measure the TEER values using a Millicell-ERS system ohm meter (Millipore, Cat# MERS 000-01).

16.1 One electrode is placed in the upper compartment and the other in the lower compartment, and the electrodes are separated by the cellular monolayer. For correction of the background resistance by subtracting the resistance value obtained with cell-free filters from the TEER of filters with MDR1-MDCKII monolayer.

- The measurement procedure includes measuring the blank resistance (R_{blank}) of the semipermeable membrane only (without cells) and measuring the resistance across the cell layer on the semipermeable membrane (R_{total}).
- The cell-specific resistance (R_{tissue}), in units of Ω, can be obtained as:

$$\mathbf{R_{tissue}\ (\Omega) = R_{total} - R_{blank}}$$

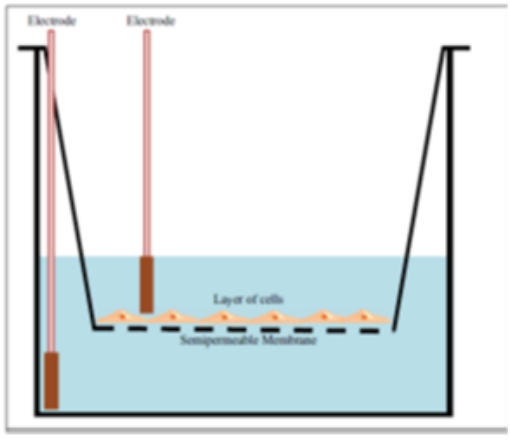
where resistance is inversely proportional to the effective area of the semipermeable membrane (M_{area}), which is reported in units of cm^2.

- TEER values are typically reported ($TEER_{reported}$) in units of Ω cm^2 and calculated as:

$$\mathbf{TEER_{reported} = R_{tissue}\ (\Omega) \times M_{area}\ (cm^2)}$$

Note

Typically, TEER values > 100 Ω cm^2 indicate adequate monolayer integrity.



17 Prepare donor solutions (containing the compound of interest) and all the reference compounds.

Note

Ketoprofen, Atenolol, and Quinidine used as reference compounds. Ketoprofen is a high permeability standard, Atenolol - is a low permeability standard, and Quinidine is a moderate-high permeability and undergoes active efflux.

18 Rinse with transport buffer 3 times the filter supports with the cell monolayers to remove residual medium by transferring the cell monolayers (medium decanted, not aspirated).

19 The MDR1-MDCKII cells pre-incubate in a transport buffer for 00:30:00 at 37 °C in both apical and basolateral compartments.

1h

- Incubate the filter inserts under gentle shaking (orbital shaker 100 rpm, 37°C, 00:30:00).

Note

Too vigorous shaking will affect the cell monolayer integrity.

20 Remove the washing solutions by decanting.

Note

Decanting instead of aspirating the washing solution reduces the risk of compromising the monolayers. Care must be taken to leave as little residual liquid as possible on the monolayers.

21 **Apical-to-basolateral (A-B) transport experiments:**

For to determine the rate of compounds transport in apical (A)-to-basolateral (B) direction, 300 µL of the test compound dissolved in transport buffer and add into the filter wells (apical compartment); 1000 µL of transport buffer add to transport analysis plate wells (basolateral compartment).

22 **Basolateral-to-apical (B-A) transport experiments:**

For to determine transport rates in the basolateral (B)-to-apical (A) direction, 1000 µL of the test compound solutions add into the wells of the transport analysis plate (basolateral compartment), the wells in the filter plate fill with 300 µL of buffer (apical compartment).

Note

The final amount of test and reference compounds [M] 10 micromolar (µM) .

23 The plates incubate for 01:30:00 at 37 °C under continuous shaking at 100 rpm .

1h 30m

- Then 75 µL aliquots taken from the donor and receiver compartments for LC-MS/MS analysis.
- All samples mix with 2 volumes of acetonitrile followed by protein sedimentation by centrifuging at.
- Supernatants are analysed using the High-performance liquid chromatography (HPLC) system coupled with a tandem mass spectrometer.

Note

All solutions of test and reference compounds were prepared manually, and further manipulations with the solutions were performed with automation using Opentrons OT-2.

CALCULATION OF PERMEABILITY COEFFICIENT

24 The apparent permeability coefficient (Papp, unit: cm s^{-1}) is determined from the amount of compound transported per time;
Papp is usually calculated according to the following equation:
Papp is expressed in 10^{-6}cm/sec.

$$P_{app} = \frac{V_A}{Area \times Time} \times \frac{[drug]_{acc}}{[drug]_{initial,d}}$$

V_A – volume of transport buffer in acceptor well,
Area – surface area of the insert (0.7 sq.cm)
Time – time of the assay,
[drug]acc – peak area of the test compound in acceptor well,
[drug]initial,d – initial peak area of the test compound in a donor well.

CALCULATION OF EFFLUX RATIO

25 Usually, perform transport experiments in both directions across the cell monolayers. The ratio between the two permeability coefficients obtained can then be used as a first indication of the involvement of an active transport process. If there is no clear difference between the permeability coefficients in the two directions, the transport

might be passive. The Efflux ratio reveals the difference in Papp as a result of active transport.
Efflux ratio is calculated according to the following equation:

$$P_{app}(BA)/P_{app}(AB)$$

Notes: The intestinal barrier is composed of multiple parallel transport processes including passive diffusion (transcellular and paracellular) and active diffusion (carrier-mediated absorption and carrier-mediated efflux). If the efflux ratio is less than 2, this indicates that the compound is passively transported. If the efflux ratio is greater than 2, this indicates the occurred active efflux.

CALCULATION OF MASS BALANCE

26 The % recovery (mass balance) can be useful in interpreting the MDR1-MDCKII data. If the recovery is very low, this may indicate poor solubility, binding of the compound to the test plate materials, metabolism by the MDR1-MDCKII cells, or accumulation of the compound in the cell monolayer.

The % recovery was calculated using the following equation:

$$\%\,recovery = \frac{C_{acc} \times V_{acc} + C_d \times V_d}{C_{initial,d} \times V_d} \times 100$$

V_{acc} – volume of compound solution in acceptor well (cm^3),
V_d – volume of compound solution in donor well (cm^3),
C_{acc} – peak area of test compound in acceptor well,
$C_{initial,d}$ – initial peak area of test compound in a donor well.

Notes: The recovery should be kept within limited ranges in order to assure high-quality and accurate P_{app} values. Limits for recovery are between 80 and 120%.

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