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P-004

# Development of a Modified Caco-2 Permeability Assay for Targeted Protein Degraders

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## Introduction

Targeted Protein Degraders (TPDs) , such as heterobifunctional degraders, and other highly lipophilic, mid-sized molecules present significant challenges in permeability assessment. In conventional in vitro permeability assays, accurate measurement of membrane permeation rates can be hindered by nonspecific adsorption and insufficient sink conditions. In addition, compounds expected to permeate biological membranes often exhibit unexpectedly low apparent permeability, making it difficult to distinguish truly non-permeable compounds from those with prolonged permeation lag times. Furthermore, compounds classified as "non-permeable" in vitro may still demonstrate gastrointestinal absorption in vivo, highlighting a gap between in vitro permeability assessment and in vivo absorption outcomes. Here, we developed an extended-incubation Caco-2 assay to evaluate compounds with prolonged permeation lag times and investigated its relationship with in vivo absorption.

## Assay development and optimization

**Hypothesis:** Some compounds classified as having low permeability under conventional conditions can be identified as permeable by extending the incubation period.

To test this hypothesis, permeability studies were conducted at three incubation time points (2, 6, and 24 h) using Caco-2 cells seeded in 96-well Transwell® plates and cultured for 10–11 days. Experimental conditions are summarized in the table 1 below.

Table 1 Experimental conditions for assay optimization

| Condition | A | B | C | D |
|---|---|---|---|---|
| Cell | Caco-2 (cultured for 10–11 days) | | | Parental MDCK |
| Donor Buffer | HBSS + 20 mM MES (pH 6.5) | | | |
| Receiver Buffer | HBSS + 20 mM HEPES + 4% BSA (pH 7.4) | | | |
| Incubation Time | 2 h | 6 h | 24 h | 24 h (Pre-incubation time) + 2 h |
| Compound Concentration | 10 µM | | | |
| Transport Direction | A-to-B | | | |

Table 2 Results under the evaluated assay conditions (TPD compounds 1 and 2 were supplied by Astellas Pharma Inc.)

| Condition \ Compound | Papp (nm/s) / Receiver concentration (µM) | | | |
|---|---|---|---|---|
| | TPD compound 1 | TPD compound 2 | Atenolol | Propranolol |
| A (conventional assay) | 2.3 / 0.0053 | 8.8 / 0.0208 | 3.6 / 0.0095 | 196.8 / 0.5525 |
| B | 2.6 / 0.0154 | 18.7 / 0.0883 | 3.4 / 0.0379 | 106.4 / 1.6093 |
| C | 5.3 / 0.1271 | 21.8 / 0.4112 | 4.6 / 0.1545 | 52.8 / 3.1944 |
| D | MDCK monolayers tended to exhibit leakage under condition D, making permeability assessment difficult. | | | |

Based on the Caco-2 results, extending the incubation period from 6 h to 24 h further increased detectable permeation (Table 2). Therefore, to investigate whether prolonged exposure could facilitate permeability assessment in a P-gp transport model, compounds were pre-incubated with MDCK cells for 24 h prior to the permeability assay. However, MDCK cells were prone to monolayer leakage during the prolonged incubation period, making permeability assessment difficult. Therefore, MDCK cells were deemed unsuitable for this assay system. The modified assay is intended primarily for compounds with low permeability in conventional assays and is not suitable for highly permeable compounds such as propranolol.

## Optimized assay conditions for TPD compounds

Assay conditions for TPD compounds were established based on the results of assay development and optimization. Although pH optimization was not performed in this study, both donor and receiver buffers were maintained at pH 7.4 to enable calculation of efflux ratios.

| Parameter | Conventional Assay | Modified Assay |
|---|---|---|
| Cell | Caco-2 (cultured for 10–11 days) | Caco-2 (cultured for 10–11 days) |
| Donor Buffer | HBSS + 20 mM MES (pH 6.5) | HBSS + 20 mM MES (pH 7.4*) |
| Receiver Buffer | HBSS + 20 mM HEPES + 4% BSA (pH 7.4) | HBSS + 20 mM HEPES + 4% BSA (pH 7.4) |
| Incubation Time | 2 h | 24 h |
| Compound Concentration | 10 µM | 10 µM |
| Transport Direction | A-to-B | A-to-B and B-to-A |
| Endpoint | Papp | Papp, efflux ratio |

*The donor and receiver buffers were adjusted to the same pH to allow accurate determination of the efflux ratio.

## Relationship analysis using in-house compounds

TPD compounds synthesized by Axcelead were used to compare the conventional assay with the modified assay.

Fig. 1 Relationship between Papp values from the conventional and modified assays

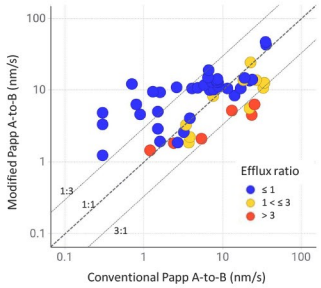



Fig. 2 Relationship between estimated FaFg and Papp obtained using the conventional assay

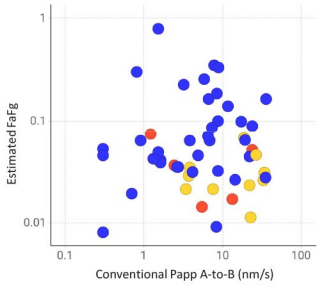
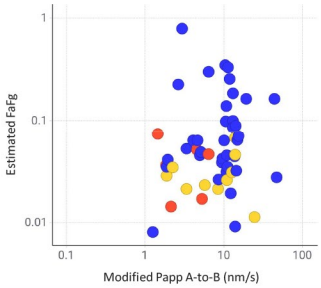



Fig. 3 Relationship between estimated FaFg and Papp obtained using the modified assay




[Mouse PK study]
All animal study protocols were approved by the Institutional Animal Care and Use Committee.
Animals
Nonfasted male ICR mice, 8 weeks old (n = 3 per dosing route)
Dose
Five compounds were administered as a cassette.
i.v. 0.1 mg/kg per compound at 1 mL/kg
p.o. 1 mg/kg per compound at 5 mL/kg
Vehicle
i.v. DMSO/1,3-butanediol
p.o. 0.5% methylcellulose solution
Plasma sampling
i.v. 5, 10, 15, and 30 min; 1, 2, 4, and 8 h
p.o. 15 and 30 min; 1, 2, 4, and 8 h
Serial blood samples were collected via tail-vein incision.
Bioanalysis
Plasma concentrations were determined by LC–MS/MS

[Estimation of FaFg]
Total clearance was assumed to be predominantly hepatic (CLtot ≈ CLh).
Hepatic availability (Fh) was approximated as 1 – CLtot/Qh using a mouse hepatic blood flow (Qh) of 90 mL/min/kg.
FaFg was estimated as BA/Fh, where absolute bioavailability (BA) was calculated from the dose-normalized AUC ratio following oral and intravenous administration.

In the modified assay, several compounds with low apparent permeability in the conventional assay exhibited increased permeability. These findings suggest that extending the incubation period improves the detection of membrane permeation in compounds with prolonged permeation lag times. Although the overall relationship between Papp and estimated FaFg remained modest, the modified assay appeared to reduce the underestimation of permeability observed for some compounds.

## Conclusion

The modified assay enabled the detection of membrane permeation in compounds that exhibited limited permeability in conventional assays, reducing the apparent underestimation of permeability observed for some compounds. The assay may be particularly useful for compounds with prolonged permeation lag times. Therefore, this approach may serve as a complementary permeability assay for TPDs and other beyond-the-Rule-of-Five compounds whose membrane permeation is difficult to detect under conventional assay conditions.

**Conflict of Interest**
This study was conducted in collaboration between Axcelead Drug Discovery Partners, Inc. and Astellas Pharma Inc., which also provided funding for the study.
Naohiro Aoyama, Koji Yokoo, and Takashi Nozawa are employees of Astellas Pharma Inc.
Takako Sono, Takashi Sasaki, Hirotaka Kamitani, and Seiji Yamasaki are employees of Axcelead Drug Discovery Partners, Inc.
The authors declare no other conflicts of interest.