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## Original Research Article

# Blood–Brain Barrier Permeability of Methyl-3,4-dihydroxybenzoate derivative NO.2 for Neurodegenerative Diseases Treatment

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E-mail: [tlhm@jnu.edu.cn](mailto:tlhm@jnu.edu.cn)ORCID: Weiyi Liu; , Jitao Hai; , Yan Luo ; Huanmin Luo; **Authors contribution:**

Weiyi Liu: participated in the whole process, including experimental operation, data analysis and manuscript writing. Jitao Hai and Yan Luo performed cell culture and analyzed data. Huanmin Luo: Conceptualization, Writing - review & editing, Supervision, Project administration.

**Core tip:**

In previous researches, we synthesized a methyl-3,4-dihydroxybenzoate (MDHB) derivative, 4-(1-(3,4-dihydroxyphenyl)-1H-1,2,3-triazol-4-yl) benzoic acid (NO.2), that presents a neurotrophic effect and was more effective than lead derivatives. NO.2 could penetrate the blood-brain barrier (BBB), indicating its potential to treat central nervous system (CNS) diseases.

**Abstract:**

**Background** The blood-brain barrier (BBB) plays a crucial role in the central nervous system (CNS) homeostasis maintenance. However, BBB limits drug entrance into the brain, leading to reduced bioavailability in the CNS. Preliminarily, a BBB *in vitro* model can help detect drug permeability and simplify the operation and reduce the initial cost of drug development. A methyl-3,4-dihydroxybenzoate (MDHB) derivative, 4-(1-(3,4-dihydroxyphenyl)-1H-1,2,3-triazol-4-yl) benzoic acid (NO.2), synthesized in our lab in previous studies, presents a neurotrophic effect and is more effective than lead derivatives. However, its BBB permeability remains unknown.

**Methods** We used Madin-Darby Canine Kidney (MDCK) cells to establish a BBB *in vitro* model to detect if NO.2 could penetrate the BBB.

**Results** The results showed that the NO.2 apparent permeability (Papp) was greater than  $0.1 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$ . Therefore, it was characterized as a moderate permeable substance that could penetrate the BBB.

**Conclusion** We presented preliminary evidence that the MDHB derivative, NO.2, could penetrate the BBB. This indicates that NO.2 has the potential to treat CNS diseases.

**Key words** Methyl 3, 4-dihydroxybenzoate; structural derivatives; blood-brain barrier; central nervous system.

## INTRODUCTION

The blood-brain barrier (BBB) is composed of highly specialized brain capillary endothelium and choroidal plexus epithelium<sup>1</sup>. They can interact with other cells, such as astrocytes and pericytes<sup>2</sup>, in the central nervous system (CNS) and play a physiological and pathological role. BBB affects disease development, forms dynamic physical and metabolic barriers, and strictly regulates the molecular exchange between the blood and the brain<sup>3</sup>. The BBB is also important to protect the brain, playing a crucial role in CNS homeostasis maintenance by transporting nutrients, clearing metabolites, and limiting the input of toxic, or harmful, molecules<sup>2</sup>. On the other hand, BBB limits drug entry into the brain, leading to reduced bioavailability in the CNS<sup>4</sup>.

The BBB *in vitro* model can be used to study drug permeability for CNS diseases treatment and simplify the operation and reduce the initial cost of drug development. Additionally, the *in vitro* model is convenient for drug screening and structure optimization, and to enhance the brain targeting of drugs<sup>5</sup>. Madin-Darby Canine Kidney (MDCK) cell model, established by Pastan *et al.* in 1988, has become one of the commonly used tools for BBB drug transport screening<sup>6,7</sup>. After around 7 days of culture under conventional cell culture conditions, MDCK cells form a tightly connected mono cell layer with a high Trans-Endothelial Electrical Resistance (TEER) value<sup>8</sup>. The experimental data obtained by this model is highly comparable to *in vivo* experiments. Therefore, the MDCK cell model is simple, efficient, stable, and its experimental results between groups are reliable<sup>9</sup>.

It has been reported that MDHB have neurotrophic activity<sup>10-12</sup>. In our previous experiments, the synthesized MDHB derivative, NO.2, had a stronger neurotrophic activity compared to lead compounds. Since the NO.2 application is intended to treat CNS disorders, its BBB permeability is crucial. Therefore, we used MDCK cells to establish a BBB model to test if NO.2 could penetrate the BBB *in vitro* and to provide more data for its future clinical use.

## MATERIAL AND METHODS

### CELL CULTURE

MDCK cells were removed from liquid nitrogen, thawed in a 37°C water bath, and transferred to a DMEM medium (2 mL) with 10% FBS. After centrifugation (1000 rpm/5 min), the supernatant was discarded and medium (6 ml) was added to resuspend the cells. Cells were inoculated in a culture flask (25 cm<sup>2</sup>) for about 2 days, then subcultured. The culture medium was drained and cells were washed with PBS two times. Then, trypsin was added (1 mL) and cells were digested in an incubator at 37°C for 10 min. After the cell edges became round, the digestion was immediately stopped by culture medium addition. Finally, cells were gently blown down, subcultured at 1:3, and continued cultured.

### MTT ASSAY

MDCK cells were inoculated on a 96-well plate (5×10<sup>3</sup> cells/well), placed in an incubator for about 18h, then replaced with a medium containing NO.2 (0.5 - 16 μM). MTT reagent (10 μL/well; Sigma) was added to each well and incubated at 37°C for 4h. After incubation, the liquid in the well was drained, and DMSO (200 μL/well; Sigma) was added to dissolve the crystal formed. After oscillation, the 490 nm absorbance was measured with a microplate analyzer.

### BBB IN VITRO MODEL ESTABLISHMENT

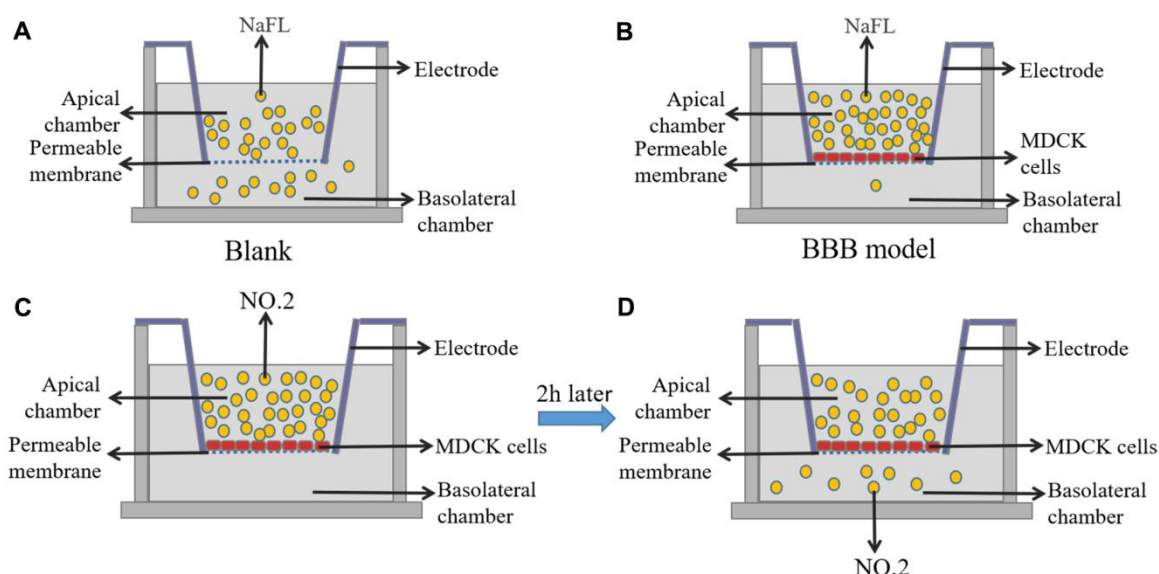
MDCK cells were inoculated into the 12-well Transwell plate upper chamber (2×10<sup>5</sup> cells/well). DMEM medium (containing 10% FBS) was added (1.5 mL) to the lower chamber. TEER was measured and recorded every day during the culture period.

### SODIUM FLUORESCIN (NAFL) LEAKAGE TEST

Sodium fluorescein (NAFL) (55 mM) was diluted with an equal concentration gradient of Hank's buffer. Fluorescence intensity was measured at  $\lambda_{Ex} / \lambda_{Em} = 428/540$  with a luciferase labeled instrument. Standard curves were drawn according to the data (Figure 3C). According to the standard curve, the NAFL linear relationship was favorable in the 0.0275 ~ 55  $\mu\text{M}$  concentration range. The media of cell models with stable TEER after 7 days were discarded. The upper and lower chambers were washed 3 times with Hank's buffer. After the last addition, the upper chamber was balanced at 37°C for 30 min. The buffer solution was drained and 0.5 mL of NAFL (55  $\mu\text{M}$ ) was added to the Transwell upper chamber (experimental group). Then, 1.5 mL of Hank's buffer was added to the lower chamber. Similarly, the corresponding solution was directly added to the Transwell upper and lower chambers without cell culture (blank control group) (Figure 1A, B). The Transwell culture plate was placed in a shaker (45 rpm) at 37°C for 2h. A hundred  $\mu\text{L}$  was sampled from the experimental and control group lower chambers and placed in the 96-well blackboard. Then, the fluorescence intensity value was determined. Finally, the apparent permeability (Papp) and the NAFL Rejection were calculated.

### NO.2 BBB PERMEABILITY TEST

Hank's buffer solution was added (1.5 mL) to the lower chambers of Transwell culture plates mentioned above, and 0.5 mL buffer solution containing NO.2 (8, 16, and 32  $\mu\text{M}$ ) was added to the upper chambers (Figure 1C, D). The Transwell culture plate was placed in a shaker (45 rpm) at 37°C for 2h. Finally, NO.2 was sampled, detected, and the Papp calculated according to the numerical values.



**Figure 1** NAFL leakage test and NO.2 transport experiment.

### APPARENT PERMEABILITY (PAPP) FORMULA

The following equation was used to calculate the Papp.

$$P_{app} = \frac{dQ}{dt \times A \times C_0}, \quad \frac{dQ}{dt} = \frac{V_r \times C_f}{dt}$$

$V_r$ : lower chamber solution volume (mL);  $C_f$ : lower chamber final compound concentration ( $\mu\text{M}$ );  $DT$ : experiment time (seconds);  $A$ : membrane surface area ( $\text{cm}^2$ );  $C_0$ : upper chamber test compound initial concentration ( $\mu\text{M}$ )

## RESULTS

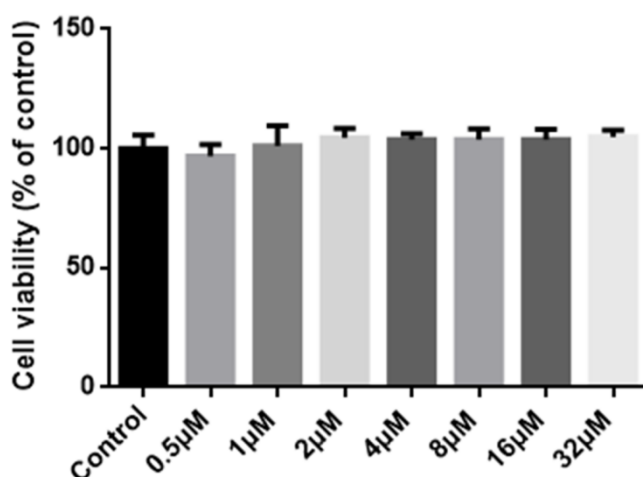
### NO.2 CYTOTOXICITY ON MDCK CELLS

MDCK cells were inoculated in 96-well plates ( $1 \times 10^4$  cells/well). After culture in an incubator for 18h, they were divided into DMSO (control) and NO.2 (0.5 - 32  $\mu$ M) groups. The MTT assay was carried out after 24 hours of continuous culture. Results showed that NO.2 (0.5 - 32  $\mu$ M) did not present toxicity to MDCK cells (**Table 1**; **Figure 2**).

**Table 1** Cytotoxicity of NO.2 determined by MTT test on MDCK cells

Group	OD value (490nm)
Control(DMSO)	$0.6376 \pm 0.0462$
NO.2 (0.5 $\mu$ M )	$0.6158 \pm 0.0337$
NO.2 (1 $\mu$ M )	$0.6464 \pm 0.0204$
NO.2(2 $\mu$ M )	$0.6723 \pm 0.0698$
NO.2 (4 $\mu$ M )	$0.6635 \pm 0.0551$
NO.2 (8 $\mu$ M )	$0.6631 \pm 0.0542$
NO.2 (16 $\mu$ M )	$0.6594 \pm 0.0381$
NO.2 (32 $\mu$ M )	$0.6671 \pm 0.0555$

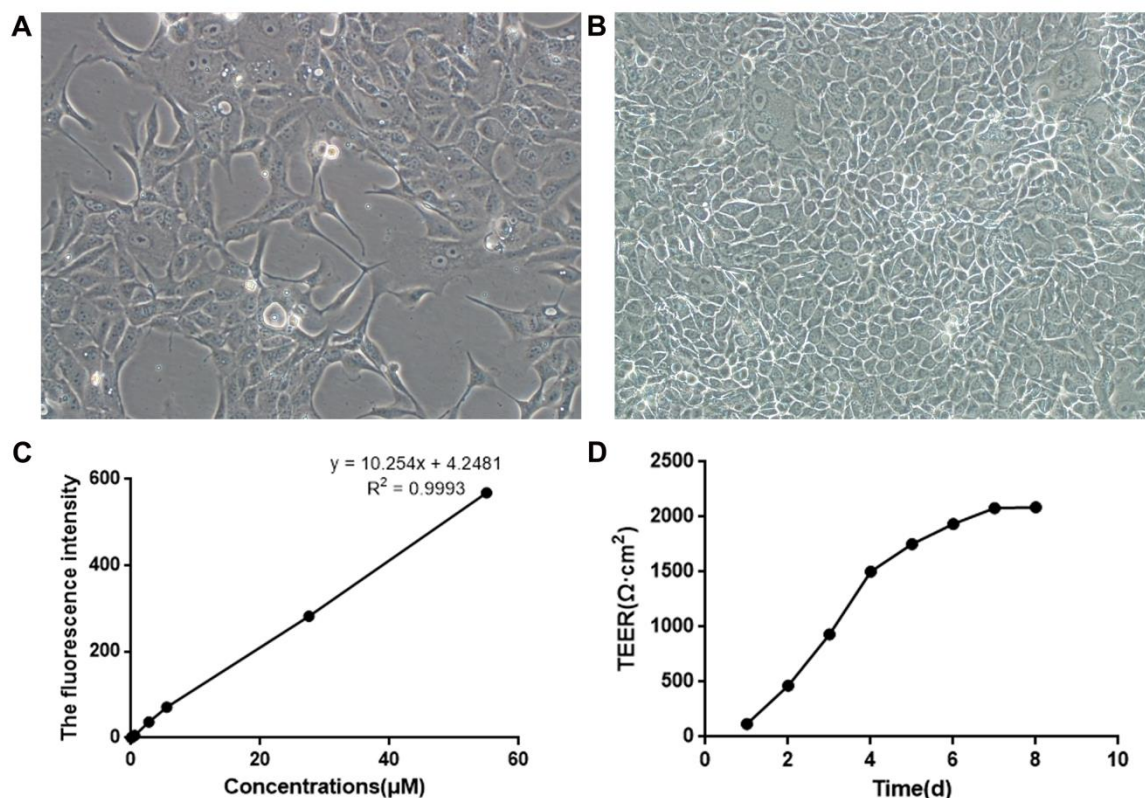
Data is presented as Mean  $\pm$  SD and n = 6



**Figure 2** NO.2 cytotoxicity on MDCK cells by MTT test.

### BBB MODEL ESTABLISHMENT

MDCK cells were inoculated in the 12-well Transwell upper compartment ( $1.5 \times 10^5$  cells/well) and 1.5 mL of medium was added into the lower compartment. The TEER was measured daily during the culture period and recorded. MDCK cells' monolayer junctions density and transport capacity were determined by the TEER value with a transmembrane resistance meter (MERS00002 type Millicell-ERS). Only monolayer cells with a TEER value greater than  $1000 \Omega \cdot \text{cm}^2$  can be used for permeability analysis<sup>13</sup>. MDCK cells showed loose intercellular junctions on the first culture day and formed a dense monolayer on the 4<sup>th</sup> day (**Figure 3A, B**). The pictures were captured by a microscope (IX-71; Olympus;  $\times 200$  magnification). With an increase in culture time, the MDCK cell model TEER value increased continuously. The TEER was superior to  $1000 \Omega$  on the 4<sup>th</sup> day and tended to stabilize on the 7<sup>th</sup> day (**Figure 3D**), indicating that the cells had formed a stable dense monolayer that could be used for the BBB permeability test.



**Figure 3** BBB model establishment. (A) MDCK cells cultured for 1 day; (B) MDCK cells cultured for 4 days; (C) NaFL standard curve; (D) TEER values recorded during MDCK cell culture.

### NAFL LEAKAGE TEST

NAFL leakage test was performed on cell models with stable TEER after 7 days. Only those with  $P_{app} < 0.5 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  could be used for permeability experiments. Control group  $P_{app}$  was  $4.32 \times 10^{-5} \text{ cm} \cdot \text{s}^{-1}$ , while for the BBB model group was  $3.83 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ , less than the  $P_{app}$  stipulated in the permeability experiment ( $0.5 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$ ) (Table 2). NaFL rejection was as high as 99.53%, indicating that, after culture for 7d, MDCK cells are closely connected on the Millicell<sup>TM</sup> membrane and a complete cell monolayer has been formed – a requirement for permeability experiments.

**Table 2** NaFL rejection test

Group	RFU(AP)	RFU(BL)	$P_{app}(\text{cm} \cdot \text{s}^{-1})$	NaFL rejection %
Blank	31980±128.69	11720±69.29	$4.32 \pm 0.69 \times 10^{-5}$	63.35±13.93
BBB model	52365±156.27	487±27.22	$3.83 \pm 0.99 \times 10^{-7}$	99.53±0.64

Data is presented as Mean ± SD and n = 3.

### NO.2 BBB PERMEABILITY TEST

A BBB *in vitro* model was successfully established to conduct NO.2 permeability experiments. The NO.2 (8 - 32  $\mu\text{M}$ )  $P_{app}$  were all greater than  $0.1 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  (Table 3).



**Table 3** Papp of NO.2 in BBB model at different concentrations

NO.2 ( $\mu\text{M}$ )	Papp ( $\text{cm}\cdot\text{s}^{-1}$ )
8	$4.54\pm0.084\times10^{-6}$
16	$3.63\pm0.757\times10^{-6}$
32	$5.32\pm0.790\times10^{-6}$

Data is presented as Mean  $\pm$  SD and n = 3.

## DISCUSSION

CNS diseases are very difficult to treat and one of the causes is the drug difficulty to cross the BBB<sup>4</sup>. Therefore, CNS drug development needs to assess BBB permeability in the early stages. Drug BBB permeability evaluation also comprehends drug Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) research - a prerequisite for CNS disorder drug treatments development<sup>5</sup>.

The use of a BBB *in vitro* model can help simplify the operation and reduce the initial cost of drug development. We used MDCK cells to establish a BBB *in vitro* model to test if the MDHB derivative, NO.2, could penetrate the BBB. Successful model preparation was confirmed by transmembrane resistance measurement and fluorescence leakage experiment. Therefore, it can be used for the NO.2 BBB permeability test. According to a literature review, the drug permeability evaluation can be divided into the following grades <sup>5,14</sup>: (1) Extremely permeable substances ( $\text{Papp} > 1\times10^{-5} \text{ cm}\cdot\text{s}^{-1}$ ); (2) Moderate permeable substances ( $\text{Papp}$  around  $0.1\sim1\times10^{-6} \text{ cm}\cdot\text{s}^{-1}$ ); and (3) Extremely difficult to penetrate substances ( $\text{Papp} < 1\times10^{-7} \text{ cm}\cdot\text{s}^{-1}$ ). Our results showed that NO.2 Papp was greater than  $0.1\times10^{-6} \text{ cm}\cdot\text{s}^{-1}$ , characterizing a moderate permeable substance that could penetrate the BBB. Altogether, our results indicated that NO.2 has the potential to treat CNS diseases.

## CONCLUSION

We used MDCK cells to establish a BBB *in vitro* model to assess if the MDHB derivative, NO.2, could penetrate the BBB. Our results showed that NO.2 Papp was greater than  $0.1\times10^{-6} \text{ cm}\cdot\text{s}^{-1}$ , characterizing a moderate permeable substance. Therefore, we presented preliminary evidence that NO.2 can penetrate the BBB, suggesting its potential in the treatment of CNS diseases.

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**Conflicts of Interest:** No conflict of interest.

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**Abbreviations:** BBB, blood-brain barrier; CNS, central nervous system; MDCK, Madin-Darby canine kidney cells; MDHB, methyl 3,4-dihydroxybenzoate; NAFL, Sodium fluorescein; NO.2, 4-(1-(3,4-dihydroxyphenyl)-1H-1,2,3-triazol-4-yl) benzoic acid; Papp, apparent permeability; TEER, Trans-endothelial Electrical Resistance

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