Use of Loperamide as a Phenotypic Probe of *mdr1a* Status in CF-1 Mice

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Purpose. A subpopulation of the CF-1 mouse strain (approximately 25%) lacks P-gp expression, and consequently, increased brain penetration of many substrates is observed in these animals. Mice lacking the *mdr1a* gene represent an important research tool to study the potential effects of P-gp on CNS substrate disposition.

Methods. Adult CF-1 mice were used in all experiments. Loperamide-induced antinociception was determined by the hotplate latency test at 0.25, 2, and 4 h post-dose. At the conclusion of the pharmacodynamic experiment(s), trunk blood and brain tissue were collected and analyzed by high-performance liquid chromatography-mass spectrometry (LC-MS/MS). Mice were also genotyped for their *mdr1a* status via RT-PCR.

Results. All mice with three consecutive effects of maximum hotplate latency (60 s) showed considerable opioid-like behavior in addition to antinociception. Mice without three consecutive effects of maximum hotplate latency (≤30 s) showed no opioid-like behavior. The loperamide brain-to-serum ratio in mice identified as P-gp–deficient was 65-fold higher compared to the P-gp-competent animals (10.1 ± 1.0 vs. 0.155 ± 0.018). All animals identified as phenotypically P-gp–competent based on the hotplate assay evidenced the *mdr1a(+/+)* genotype.

Conclusion. This assay appears to offer a rapid and unambiguous measure via a relatively non-invasive, simple technique to identify P-gp status in the CF-1 subpopulation of mice.

KEY WORDS: antinociception; CF-1 mice; P-glycoprotein; phenotyping; loperamide.

INTRODUCTION

P-glycoprotein (P-gp) is the prototypical multidrug resistance (MDR) transport protein. Originally identified based on an ability to impart drug resistance to cancer cells (1), P-gp is by far the most well characterized of the blood-brain barrier (BBB) efflux transport systems (2). P-gp is a 170-kDa energy-dependent plasma membrane efflux protein and a member of the ABC superfamily of transport systems (3,4). Experiments performed in mice that lack P-gp expression [e.g., *mdr1a (–/–)* animals] have suggested that the transporter is an important determinant of substrate delivery across the BBB. Although *mdr1a(–/–)* mice do not display a decreased life span and are fertile, they do evidence a marked increase in brain uptake of numerous drugs and other xenobiotics, with a concomitant increase in centrally-mediated pharmacologic response (2), consistent with the absence of P-gp at the blood-brain interface.

There are a variety of models available to study the impact of P-gp on drug disposition. *In vitro* models such as cultured brain microvessel endothelial cells (BMECs) are good for identifying mechanisms of transport across the BBB, but as they cannot imitate the dynamic nature of the BBB, the *in vitro* values recovered often do not correlate to an *in vivo* situation. In order to get a more accurate depiction of *in vivo* CNS disposition, animal models (either transgenic or naturally occurring mutants) are most often used. A subpopulation of the CF-1 mouse strain (approximately 25%) lacks P-gp expression. Consequently, increased brain penetration of many substrates is observed in these animals. The genetic level of this mutation has been established, and studies have revealed that this inheritance follows a normal Mendelian autosomal pattern (5). The DNA appears to be stable over time and there appears to be no selection process for or against the mutant alleles [i.e., no selection pressure with the P-gp-deficient mice in the absence of drug treatment (6)]. In addition, the mutant mice appear to be deficient in P-gp in those tissues that express predominantly the *mdr1a* isoform (i.e., brain and intestine), indicating that this deletion is restricted to the *mdr1a* gene only (6). There are three genes that encode different isoforms of P-gp in mice: *mdr1a* (also known as *mdr*3), *mdr1b* (also known as *mdr*1), and *mdr2* (7). The multidrug resistant phenotype is associated with both mdr1a and mdr1b in mice, while mdr2 is necessary for bile production. Mice lacking the *mdr1a* gene represent a valuable research platform with which to study the potential effects of P-gp on substrate disposition, especially with regards to the CNS. However, while these animals (spontaneous naturally occurring mutants) offer the advantage of a lower cost than transgenic (knockout) animals, it is imperative that the investigator know which animals are phenotypically P-gp–competent or P-gp–deficient. The two substrains of mice are virtually identical with regards to gross physiology; spontaneously mutated animals (misidentified as P-gp-competent when they are, in fact, P-gp-deficient) typically are excluded from post-experimental analysis as statistical outliers. Therefore, development of an inexpensive, quick and efficient method to phenotype mice for P-gp function would provide distinct advantages.

In this paper, we describe a simple approach for phenotyping mice for BBB P-gp function. This method is benign for P-gp–competent animals and relatively innocuous even in P-gp–deficient mice.

MATERIALS AND METHODS

Chemicals

Loperamide hydrochloride, (±)-methadone hydrochloride, ribonuclease A (RNase A), and proteinase K were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were the highest grade available from commercial sources.

Animals

Adult CF-1 mice [*mdr1a(+/+)* and *mdr1a(–/–)*, 25–30 g, 6–8 weeks of age] were purchased form Charles River Laboratories (Wilmington, MA, USA) and maintained in a breeding colony in the School of Pharmacy, The University of North Carolina. Male and female mice were housed sepa-

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rately (maximum of 4 per cage) in wire-mesh cages in a temperature- and humidity-controlled room with a 12-h dark/12-h light cycle and had unrestricted access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of North Carolina, and all procedures were conducted according to the "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, revised in 1985).

Behavioral and Dispositional Phenotyping

Mice (n = 18; with the experimenter blinded to purported phenotype) received a 2-mg/kg subcutaneous (s.c.) dose of loperamide prepared as a solution in 50% propylene glycol. At 0.25, 2, 4, and 24 h post-dose, mice were observed for signs of opioid intoxication [i.e., Straub reaction (8)], and loperamide-induced antinociception was determined by the hotplate latency test as previously described (9). Briefly, latency was defined as the time interval between the placement on the hotplate (55°C; Columbus Instruments, Columbus, OH, USA) and the licking of the hind paws or jumping. To avoid tissue damage, a maximum test latency of 60 s was used. Baseline latency was determined prior to administration of loperamide.

Following a 2-week wash-out period, mice received a second 2-mg/kg s.c. dose of loperamide, and signs of opioid intoxication and hotplate latencies were determined as after the first dose. The animals were decapitated, and trunk blood and brain tissue were collected following the 4-h observation. Blood was allowed to clot at room temperature for least 30 min before centrifuging to collect serum. Brain and serum samples were stored at –20°C until analysis by LC-MS/MS.

Quantitation of Loperamide in Serum and Brain

Brain samples were homogenized in water (1:2 v/v) via sonic probe. A 25-μl aliquot of homogenate or plasma was transferred to an HPLC vial, and protein was precipitated with 250 μl methanol containing internal standard (methadone, 20 ng/ml). The sample was vortex-mixed, centrifuged, and the supernatant was analyzed by LC-MS/MS. Samples were injected (3 μl; Agilent 1100 wellplate autosampler) onto a Phenomenex 2.0 × 30 mm 4 μm Synergi Max-RP column (Phenomenex, Torrance, CA, USA) maintained at room temperature. Analytes were eluted with a linear gradient (750 μl/min) consisting of ammonium acetate (pH 6.8; 10 mM) ["A"] and methanol ["B"] produced by an Agilent 1100 series binary pump. An initial concentration of 20% "B" was ramped to a final concentration of 95% over 2 min and held for 1 min. The system was returned to the initial condition in a single step and allowed to equilibrate for 1 min. The entire column effluent was diverted from the Turbo Ionspray of a PE-Sciex API-4000 triple quadrupole mass spectrometer for the first and last min. Loperamide and methadone were measured using multiple reaction monitoring (477.4→266.0 and 310.3→265.1, respectively). Standard curves were prepared in brain homogenate and plasma.

Genotyping of CF-1 Mice

After sacrificing, the distal ~1.5 cm of the tail was removed. Tail tissue was minced and incubated overnight in 250 μl digestion buffer (50 mM Tris, 100 mM NaCl, 10 mM EDTA, 1% SDS, 0.5 mg/ml proteinase K; pH 8; 55°C). Following digestion, 1.5 μl of RNase (10 mg/ml) was added to each sample, which was incubated at 37°C for 20 min. For DNA extraction, 160 μl of 5 M ammonium acetate was added, samples were mixed by vortex briefly, and centrifuged (16,000 × *g*, 4 min). The supernatant was poured into 600 μl isopropanol (4°C), gently mixed, and centrifuged (16,000 × *g*, 7 min). The supernatant was discarded and 600 μl ethanol (70%; 4°C) added. Following centrifugation (16,000 × *g*, 4 min), the supernatant was removed, the pellet was air-dried for ~15 min and suspended in TE buffer (20 μl; 10 mM Tris, 1 mM EDTA, pH 8).

DNA enrichment was conducted with Herculase Enhanced DNA polymerase kit (Stratagene, La Jolla, CA, USA) and dNTP Mix (Amersham Biosciences, Piscataway, NJ, USA) with the primers 5′CTTTGACTCGGGAGCAGAAG3′ (forward) and 5′GAATGAACTGACCTGCCCCA3′ (reverse) (UNC Nucleic Acids Core Facility) (10). Following an initial cycle at 94°C for 2 min, polymerase chain reaction (PCR) was conducted for 35 cycles (94°C for 30 s, 60°C for 30 s, 68°C for 10 min). The resulting PCR products were extracted and precipitated as noted above, except that samples were stored at –80°C for 20 min following isopropanol addition to improve precipitation. The final pellet was dissolved in TE buffer and stored at 4°C.

A second PCR to isolate the *mdr1a* gene region of interest was conducted using the same conditions as above and the primers 5′CCAGAGCTTGCAGATACCAT3′ (forward) and 5′CACGTGTGCTTTCTTCATCG3′ (reverse). The resulting products were run on a 1% agarose gel and stained with ethidium bromide. Products were visualized on a VersaDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Approximate molecular weights for the PCR products were determined using a GeneRuler 1kb DNA Ladder (Fermentas Inc., Hanover, MD, USA).

Data Analysis

Where appropriate, a two-tailed Student's *t* test was used to evaluate the statistical significance of differences between experimental groups. In all cases, $p < 0.05$ was used as the criterion of statistical significance.

RESULTS

Behavioral Phenotyping

All mice with three consecutive effects of maximum hotplate latency (60 s; at 0.25, 2 and 4 h post-dose) showed considerable opioid-like behavior in addition to antinociception, which persisted at least through 4 h (Fig. 1). These behaviors included hunched posture, compulsive circling, decreased coordination, and the classic Straub tail reaction (rigid erect tail) (8). Mice that did not display three consecutive effects of maximum hotplate latency (60 s) showed no opioid-like behavior, and each of these mice displayed a hotplate latency ≤30 s. No opioid-induced behaviors were noticeable 24 h post-dose in any of the mice.

Loperamide Brain and Serum Concentrations

The loperamide serum concentration in the mice identified during the phenotyping phase of the experiment as P-gp–

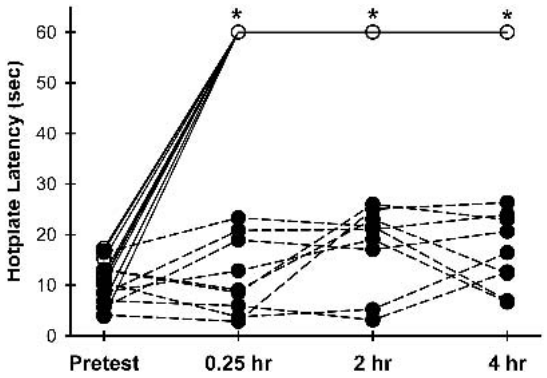

Fig. 1. Results of the hotplate latency test following a 2-mg/kg s.c. dose of loperamide. ● indicates mice that were phenotypically *mdr1a(+/+)*, and ○ indicates mice that were phenotypically *mdr1a(–/–)*. *p < 0.01 vs. pretest.

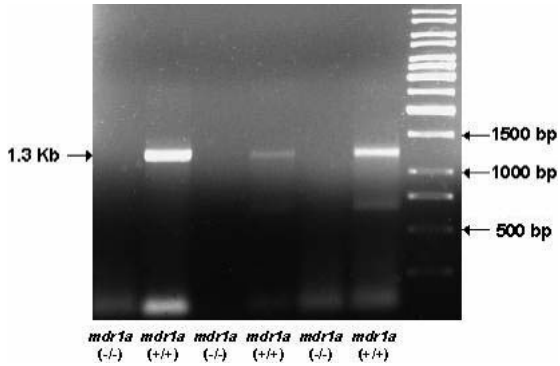

Fig. 3. Representative RT-PCR gel indicating the presence of a 1.3-kb product only in those mice that were phenotyped/genotyped *mdr1a(+/+)*.

competent was 50 ± 28 ng/ml (mean ± SD). The loperamide brain concentration in these same mice was 6.7 ± 2.8 ng/g (mean ± SD) The loperamide serum concentration in the mice identified during the phenotyping phase of the experiment as P-gp–deficient was 50 ± 29 ng/ml (mean ± SD). The loperamide brain concentration in these same mice was 468 ± 158 ng/ml (mean ± SD). The loperamide brain-to-serum ratio in mice identified during the phenotyping phase of the experiment as P-gp–deficient was 10.1 ± 1.0 (mean ± SE), whereas the loperamide brain-to-serum ratio in mice identified as P-gp-competent was 0.155 ± 0.018 (mean ± SE), representing a 65-fold higher loperamide brain-to-serum ratio in the absence of P-gp-mediated transport (Fig. 2). With respect to the identification of P-gp function utilizing a pharmacologic endpoint, no false negatives (Region I in Fig. 2) or false positives (Region IV 2 Fig. 2) were encountered among the 18 animals phenotyped. In addition, all animals that were identified as phenotypically P-gp–competent based on the hotplate assay evidenced the *mdr1a(+/+)* genotype (Fig. 3).

DISCUSSION

Loperamide is an opioid drug used clinically as an antidiarrheal that normally does not cross the BBB (due to P-gp–mediated efflux) and therefore lacks opiate-like effects in the CNS of animals or man (11). The large P-gp effect (i.e., dependence of the degree of brain uptake on P-gp–mediated efflux) and obvious end-points for centrally mediated pharmacological activity indicated that loperamide may be a useful probe substrate for phenotyping the CF-1 mice (12). Also, recent clinical studies have used loperamide for investigating the polymorphisms in the MDR1 gene in humans (13,14). In addition, the choice of loperamide as a phenotyping probe substrate offers many advantages over the previously suggested use of the neurotoxin avermectin (5). The dose-response curve for avermectin is much more steep (15) than loperamide and the endpoint with avermectin is neurotoxicity, which may make those animals that display neurotoxicity [thus, *mdr1a(–/–)*] unattractive for future studies exploring the impact of P-gp on CNS disposition. On the other hand, loperamide appears to be harmless to the mice, and the opiate-like behavior (much less severe than the tremors experienced with avermectin toxicity) produced in P-gp–deficient, but not P-gp–competent animals dissipates within 24 h. Thus, phenotyping with subsequent experimentation can be performed after a minimum washout period, using the same mice without fear of compromising the experiment. In addition, loperamide is relatively inexpensive and easy to obtain and use.

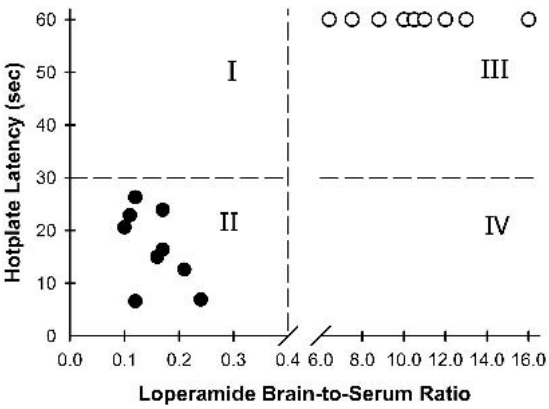

Fig. 2. Comparison between the hotplate latency results and the loperamide brain-to-serum ratio following a 2-mg/kg s.c. dose of loperamide (4 h). ● indicates mice that were phenotypically *mdr1a(+/+)*, and ○ indicates mice that were phenotypically *mdr1a(–/–)*.

The current experiments confirmed the *mdr1a* phenotype can be determined with a single 2-mg/kg s.c. dose of loperamide (Fig. 1). Although the hot plate assay offers an objective measurement of difference in behavior between P-gp–deficient and P-gp–competent mice following the administration of loperamide, the visual cues indicated by defined behavioral endpoints (i.e., hunched posture, compulsive circling, decreased coordination and the Straub tail reaction) provided an alternative end-point that was obvious and not dependent on instrumentation. On the other hand, mice that did not display three consecutive effects of maximum hotplate latency (60 s) showed no opioid-like behavior, and each of these mice displayed a hotplate latency ≤30 s. No opioid-induced behaviors were noticeable 24 h post-dose in any of the mice.

In addition to assessing the behavioral effects associated with loperamide administration, loperamide brain-to-serum ratios were determined to confirm that mice identified as P-gp-deficient [presumably *mdr1a*(–/–)] based on pharmacological activity evidenced increased brain penetration of loperamide (i.e., were phenotypically P-gp–deficient based on

transporter function). The loperamide brain-to-serum ratio was increased 65-fold in those mice identified as P-gp–deficient compared to the P-gp–competent. Also, no false negatives or positives were encountered during the experiment, indicating that this approach appears to provide an accurate identification of P-gp status.

Finally, the mice were genotyped to confirm the presence or absence of the *mdr1a* gene. All animals that were identified as phenotypically P-gp–competent based on the hotplate assay evidenced the *mdr1a(+/+)* genotype (Fig. 3). The subpopulation of CF-1 mice that evidences P-gp deficiency is due to a truncated mRNA with a deleted exon 23 resulting from an insertion at the exon 23 intron-exon juntion corresponding to a murine leukemia virus (10). Thus, this subpopulation of CF-1 mice represents an instance where a spontaneous insertional mutagenesis event produces a stable germ-line mutation (10). The fact that this method allows you to determine genotype based on behavioral phenotyping is another advantage of this method as genotype and phenotype do not always correlate.

Though increased substrate uptake into brain has been shown in the presence of chemical inhibitors of P-gp–mediated transport, pharmacokinetic experiments in transport-deficient mice form the foundation of the current understanding of attenuated BBB translocation by P-gp. Use of these animals is imperative to furthering the development of CNS agents, and an efficient means of phenotyping the mice should prove useful for studying the role of P-gp in drug disposition. Not only is it necessary to know the phenotype of the animals that are to be part of an experiment, it is also essential to know the genotype of the animals that you place together as breeders for colony maintenance. Though it is true that misidentified animals can be excluded from data analysis as statistical outliers, relying on this causes unnecessary loss of compound and animal manipulation. On the other hand, genetic tests are also available to genotype the animals, similar to the genotyping experiments performed here to confirm our results. However, these tests are dependent on the ability to perform the tests or the cost associated with sending samples off-site to have them tested. Also, genetic tests require certain skill sets, equipment, cost and are much more time consumptive than the method presented herein. Thus, this method appears to offer an alternative clear and rapid method of phenotyping the subpopulation of CF-1 mice that is deficient in P-gp.

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