Surface Plasmon Resonance Studies of the Direct Interaction Between a Drug/Intestinal Brush Border Membrane

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Purpose. We describe here a new method to estimate the oral drug permeability from the small intestine using surface plasmon resonance (SPR) technology. The interaction between drugs and brush border membrane (BBM) surfaces immobilized on biosensor chip were evaluated by measuring the SPR response signal.

Methods. BBM vesicles, isolated from Sprague-Dawley (SD) rats, were immobilized onto the L1 chip composed of dextran derivatives with modified lipophilic residues. A SPR (BIAcore 3000) was used with L1 chip, and it was carried out in a running buffer, HEPES-buffered saline containing 0.1% DMSO. Fourteen drugs for the SPR experiments were flowed over the BBM immobilized L1 chip, and the response levels according to the BBM surfaces were evaluated directly in a continuous flow system.

Results. The immobilized BBM surface on L1 chip was very stable, and it was regenerated by injecting a new BBM vesicle solution. It was evident that drug binding events, using BBM surfaces, directly provides information that predicts the F_a value in human for transcellularly absorbed drugs. The throughput to assay each drug at a single concentration is 100 drugs for 24 h.

Conclusions. The interaction between drugs and small intestinal surfaces was successfully assayed using SPR technology, and this SPR analysis exhibited advantages: lack of labeling requirements, the real-time acquirement of various results, and the repeated use for various drugs.

KEY WORDS: drug fraction absorbed; intestinal brush border membrane; oral drug permeability; surface plasmon resonance.

INTRODUCTION

The primary objective of permeability screening assays in drug development is to predict the fraction of drug absorbed (F_a) from the small intestine. On passive and/or active transport, most drugs developed for oral administration are absorbed in the gastrointestinal tract followed by systemic circulation. Ideally, the intestinal permeability of a drug could be quantified *in vivo* by directly measuring the F_a value in animals and humans. The F_a value also has been measured using *in vitro* screening assays; for example, Caco-2 cells (1), tissue diffusion (2), liposome chromatography (3), lipid or brush border membrane (BBM) vesicles partitioning (4,5), and computational methods (6,7). But these *in vivo/in vitro* permeability screening assays are time-consuming, laborious, and somewhat difficult to perform high-throughput screening. In particular, they need further analytical procedures where the final detection is based on UV spectroscopy, mass spectrometry, or radiometry of labeled compounds.

Recently, SPR has been used for an optical detection system that allows a direct analysis of the interaction between the drugs and phospholipid immobilized surfaces on biosensor chips in a continuous flow system (8–10). In this system, lipid surfaces, prepared by vesicle fusion on a biosensor chip, could mimic the biomembranes of intestinal epithelial cells, and also the microfluidic flow system could evaluate the real-time interaction between drugs and lipid surfaces without the need for intrinsic or extrinsic labels. Although the quantitative measurement of the passive transport is readily predicted using lipid immobilized SPR system, the estimation of the active transport has been hindered because of the presence of various carrier proteins in intestinal membrane. Moreover, the lipid immobilized SPR system showed different interactions with orally absorbed drugs according to physicochemical properties of lipids, such as lipid structure, lipid ordering, and lipid fluidity (10).

To solve these problems, we previously studied a new SPR system to estimate binding events occurring on the intestinal membranes using BBM immobilized biosensor chips (11). Because BBM vesicles possess the same structural and functional similarity to that of real intestinal membrane, they can be effectively used to estimate the passive and/or active transport mechanism of drugs and nutrients (12,13). This BBM immobilized SPR system has demonstrated that the calcium channels and bile acid transporters can be evaluated by flowing the calcium channel blocker and bile salts over the biosensor chip. Therefore, BBM immobilized biosensor chip could be used to estimate the absorption of drugs and nutrients that may be orally administrated because of their structural and functional similarity to real intestinal membrane containing phospholipids, hydrolytic enzymes, and carrier proteins.

In this study, we further expanded SPR studies for the estimation of the fraction of drug absorbed in the human intestine. In an attempt to mimic the real intestinal surface capable of interacting with drugs, we immobilized BBM surfaces on the biosensor chip, and the interaction between drugs and BBM surfaces was monitored directly by measuring the changes in refractive index at the sensor surface caused by changes in mass (Fig. 1). The detection of the interaction between drugs and BBM surfaces does not therefore require chromophoric or radiolabeled compounds, as do some other methods.

MATERIALS AND METHODS

Materials

All drugs, bovine serum albumin (BSA), and DMSO were purchased from Sigma (St. Louis, MO, USA). The L1 sensor chip was obtained from BIAcore AB (Uppsala, Sweden). All other reagents were obtained from Merck (Darmstadt, Germany) or Sigma.

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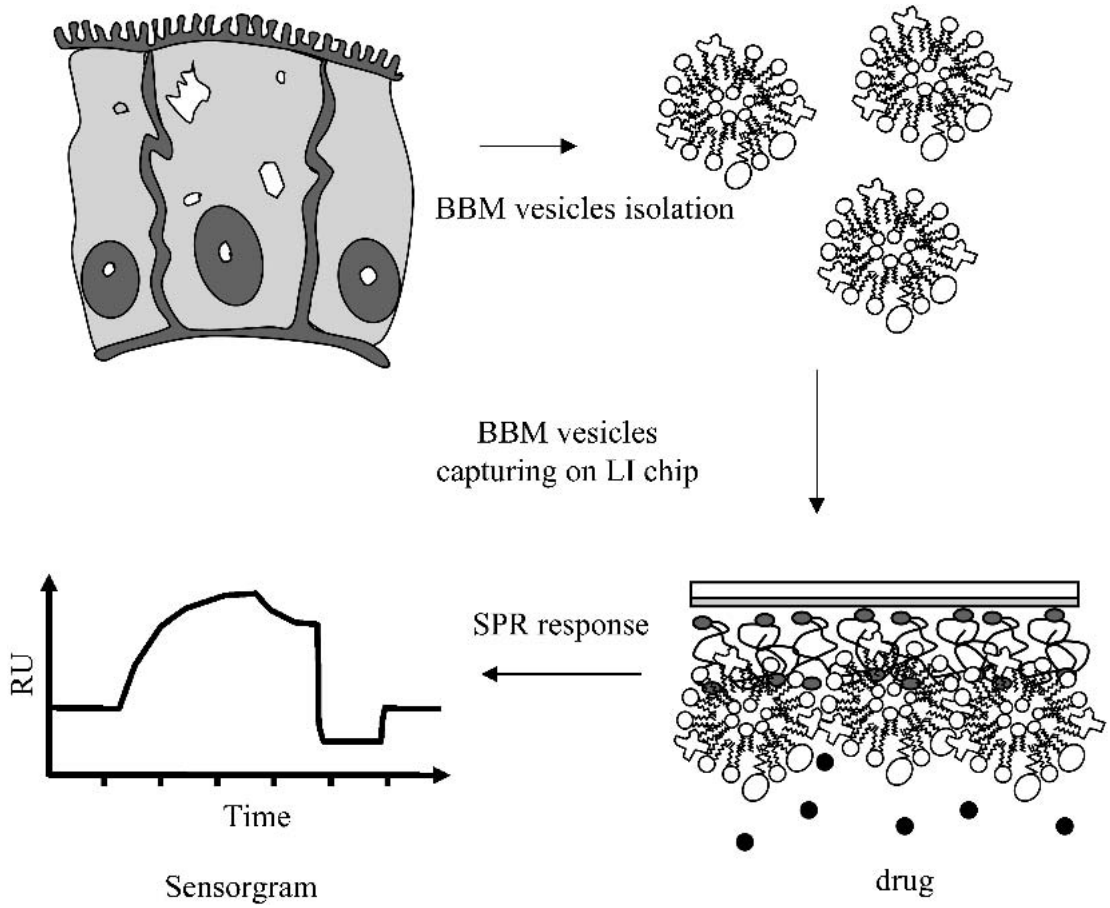

Fig. 1. Schematic diagrams for BBM vesicles isolation from small intestine, BBM vesicles capturing on L1 chip, and monitoring the interaction drugs and BBM surfaces.

Drug Solution Preparation

The drugs were dissolved in DMSO (stock 20 mM) or directly in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 1% DMSO (20 mM) depending on their respective solubility. The bulk solutions were carefully diluted with HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) to range from 500 μM to 16.5 μM, and the DMSO concentration in bulk solution was carefully matched to 0.1%.

Preparation of BBM Vesicles

BBM vesicles from small intestines of SD rats (200–220 g, fasted overnight) were prepared by the Ca^{2+} precipitation method, as described previously (11). The entire small intestine was removed and divided into 10 segments of equal length from proximal to distal. All segments were used for the preparation of small intestine BBM vesicles. The BBM vesicles were enriched (5, 2.5, 1, 0.5 mg/ml) with respect to BBM vesicles' protein concentration determined by micro-BCA assay, and the purity of BBM vesicles was determined by established procedure of an activity assay for alkaline phosphatase (14) and Na^+K^+ ATPase (15). Immediately after preparation, the BBM vesicles were used for the SPR experiments within 6 h without loss of carrier proteins and enzymatic activity of BBM vesicles for at least 1 day (11,16). After all experiments, we immediately checked the bioactivity of BBM vesicles to maintain the same property of BBM vesicles during SPR experiments.

Immobilization of BBM Surfaces on the L1 Chip

BBM vesicles were isolated from small intestine and immobilized on the L1 chip using SPR technology. Prior to use, the L1 chip surface was washed with an injection of 40 mM 3-[(3-cholamidopropyl) dimethylamonio]-1-propanesulfonate (CHAPS) at a flow rate of 5 μl/min for 4 min, followed by washing with a running buffer at a flow rate of 5 μl/min for 4 min. Then, each BBM vesicle solution with different protein concentrations from 0.5 mg/ml to 5 mg/ml was injected at a flow rate of 2 μl/min for 2.5 min, and the running buffer was allowed to flow continuously to remove any slightly absorbed BBM vesicles. Finally, the response level according to the immobilized BBM vesicles was equilibrated within 15 min. The surface coverage of BBM vesicles on L1 chip was evaluated using a bovine serum albumin (BSA) (0.5 mg/ml) binding assay on L1 chips and BBM vesicle immobilized L1 chips.

SPR Experiments

A BIAcore 3000 (Biacore AB, Uppsala, Sweden) was used with L1 sensor chips. All SPR experiments were carried out in a running buffer, HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 0.1% DMSO. Each drug for the SPR analysis was dissolved in a running

buffer at predetermined concentrations, and it was injected at a flow rate of 20 μl/min for 2 min. Drug injections were repeated three times during each experiment and run in random order. After the analysis of each drug, the L1 chip was readily regenerated by an injection of 100 mM HCl/isopropanol (50/50 v/v) solution at a flow rate of 20 μl/min for 2 min. All data were double-referenced to correct for bulk refractive index changes and systematic artifacts observed between BBM surfaces and the reference flow cell. All data were analyzed using BIAevaluation software (Biacore AB, Uppsala, Sweden).

RESULTS AND DISCUSSION

Formation of BBM Surfaces on L1 Chips

BBM surfaces were produced on the L1 chip by the injection of BBM vesicle solutions with different protein concentrations (0.5–5 mg/ml). Before BBM vesicles injection, the L1 chip was conditioned to ensure that the surface was free of any contaminants by washing with CHAPS solution and followed by running buffer. Then, BBM vesicles were immobilized on a single flow cell of L1 chip by injecting 5 μl of each BBM vesicle solution at the rate of 2 μl/min. Figure 2 presents the propensity for the formation of BBM surfaces on L1 chips to different BBM vesicles concentrations. The resonance response of BBM surfaces increased from 1362 ± 46 to 3895 ± 166 resonance units (RU) according to increasing the concentration of BBM vesicle solution from 0.5 mg/ml to 5 mg/ml. Thus, different amounts of BBM vesicles were immobilized on the L1 chip surface by varying the concentration of BBM vesicle solution.

The surface coverage of BBM surfaces on the L1 chip was confirmed by the extent of nonspecific binding of BSA, which binds significantly to the L1 chip surface composed of dextran matrix modified with lipophilic moiety (11,17). Figure 3 shows the binding patterns of 40 μl of BSA (0.5 mg/ml) to the L1 chip and BBM surfaces at a rate of 20 μl/min. BSA injection to the L1 chip resulted in a high increase in the resonance signal (650 ± 25 RU) at the end of injection. But, the degree of nonspecific binding of BSA to BBM surfaces decreased significantly, compared to the bare L1 chip. For the BBM vesicles concentration above 2.5 mg/ml, the BSA binding minimized to the 54 ± 0.8 RU. This indicates that the BBM surface was fully covered on the L1 chip surface by injecting 2.5 mg/ml of BBM vesicle solution at the flow rate of 2 μl/min for 2.5 min. The condition of BBM surface immobilization was set up by injecting the 2.5 mg/ml of BBM vesicle solution in a running buffer at 25°C.

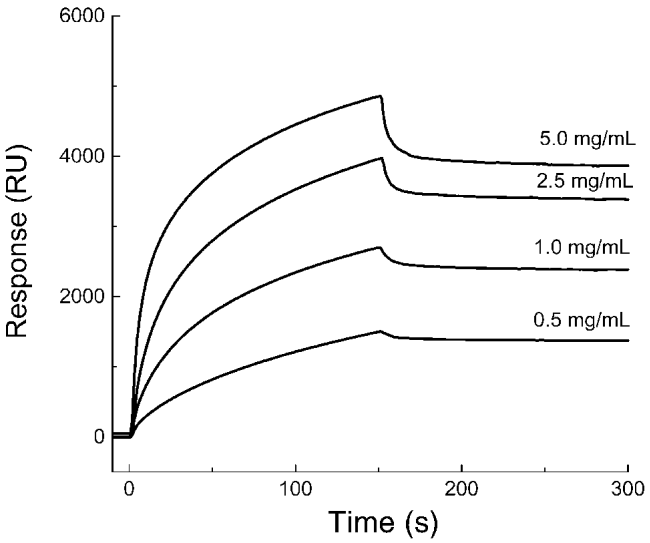

Fig. 2. Loading of the L1 sensor chip with BBM vesicle solutions. Five microliters of BBM vesicle solution with different protein concentrations (5, 2.5, 1, and 0.5 mg/ml) was injected at a flow rate of 2 μl/min.

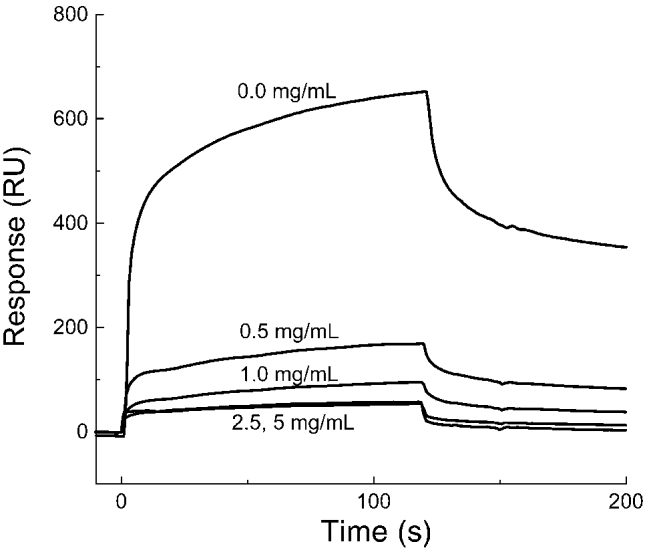

Fig. 3. Binding of albumin on an L1 chip and BBM vesicles (5, 2.5, 1, and 0.5 mg/ml) captured surfaces. Forty microliters of albumin was injected at a flow rate of 20 μl/min.

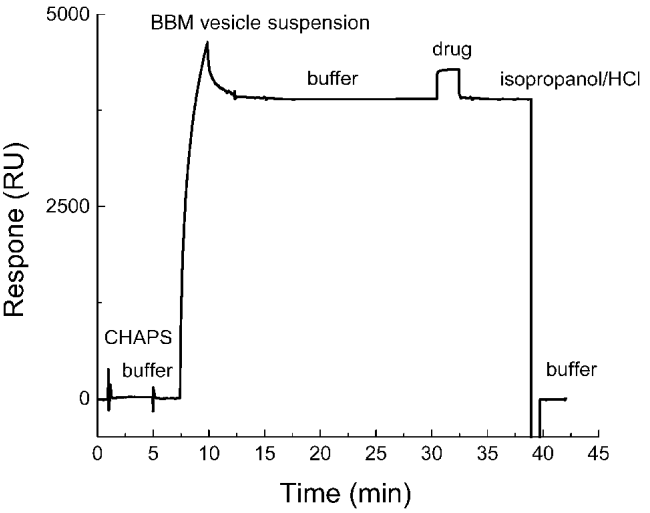

Fig. 4. The complete cycle of a sensorgram of the analysis of BBM/drug interaction. A stable BBM surface was produced by successive injections of 40 μl of CHAPS followed by 40 μl of buffer. BBM vesicle suspension with the protein concentration of 2.5 mg/ml was then captured, and buffer was injected to remove any free BBM vesicles. Then, the immobilized BBM surface was stabilized for 20 min. Forty microliters of drug was then injected over the BBM surface at the rate of 20 μl/min. Finally, the L1 chip was regenerated by injection of a 50/50 (v/v) mixture of 100 mM HCl and isopropanol followed by injection of buffer to remove any residual regeneration solution.

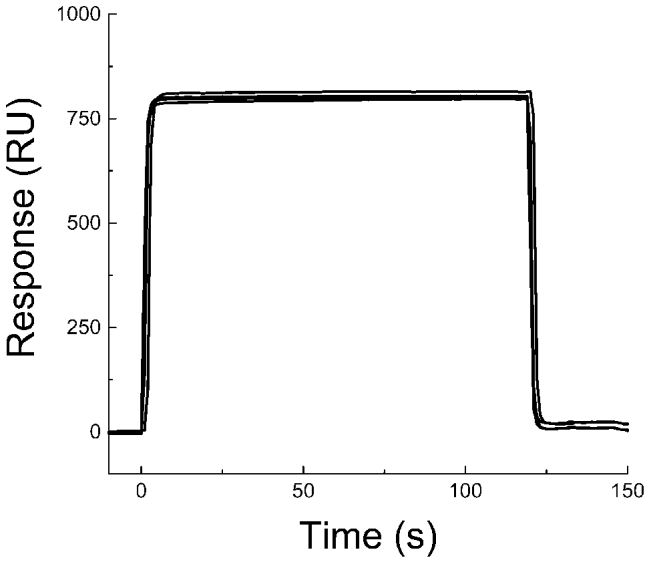

Fig. 5. Reproducibility of the response signal obtained for pindolol binding to BBM surfaces. Pindolol was injected over a BBM surface at a concentration of 500 μM, and responses were obtained from five independent experiments.

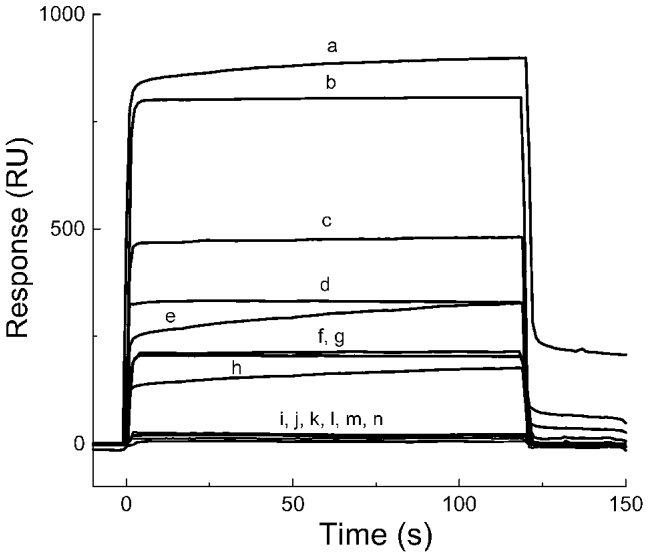

Fig. 6. Relative SPR responses for drugs to BBM surfaces: (a) desipramine, (b) pindolol, (c) ketoprefen, (d) sulfasalazine, (e) metoprolol, (f) naproxen, (g) verapamil, (h) hydrochlorothiazide, (i) atenolol, (j) creatinine, (k) D-glucose, (l) urea, and (m) mannitol were injected over a BBM surface at a concentration of 500 μM and responses were obtained.

Drug Binding Responses to BBM Surfaces

After the BBM surface immobilization, the L1 chip and microfludic system were washed with a running buffer to remove any free BBM vesicles and slightly absorbed BBM vesicles. After this washing step within 30 min, the response signal of BBM surfaces maintained to 3895 ± 166 RU with a drift of 0.5 RU/min. This optimized binding level of BBM surfaces was reproducible and independent of the change of L1 chip and BBM vesicle solution. Then, each drug was injected to the BBM surface at a rate of 20 μl/min for 2 min, and it gave characteristic response according to the interaction of a drug/BBM surface. After the drug screening, the BBM surface was completely stripped off with a 40 μl injection of a 50/50 (v/v) mixture of 100 mM HCl/isopropanol. This optimized drug assay was automatically set up to repeat the BBM vesicle capture, drug assay, and BBM surface regeneration over 50 times per each flow cell of L1 chip. The complete sensorgram obtained from a typical binding cycle, which includes BBM vesicles capture, drug binding, and regeneration, is shown in Fig. 4.

In order to perform a reliable screening assay, each drug concentration was repeated and the order of injection randomized. It has been known that replicate experiments provide an independent assessment of the total experimental noise in SPR experiments, and randomizing the samples is essential for removing any propensity in the results (18).

Table I. Drug Classifications and Response Signals from the BBM Surfaces

Drug	F_a(%)*	Transp†	MW	Response signal
Desipramine	100	t	266	898 ± 23
Pindolol	92	t	314	802 ± 7.3
Ketoproefen	100	t	254	480 ± 5.9
Sulfasalazine	12	t	398	326 ± 5.8
Metoprolol	95	t	267	325 ± 6.2
Naproxen	100	t	230	200 ± 5.7
Verapamil	100	t	455	174 ± 4.2
Hydrochlorothiazide	55	t	298	20 ± 1.5
Atenolol	54	t	266	15 ± 1.2
Creatinine	100	c, t	113	12 ± 1.1
D-glucose	100	c	180	5.3 ± 0.6
Urea	100	p	60	5.2 ± 0.8
Mannitol	26	p	182	5.1 ± 0.7

Response signal data were reported as mean ± SD for 3 time injections. BBM, brush border membrane.

* F_a(%): fraction absorbed in humans (Ref. 8).

† Transp, transport mechanism; t = passive transcellular, p = passive paracellular, c = carrier mediated.

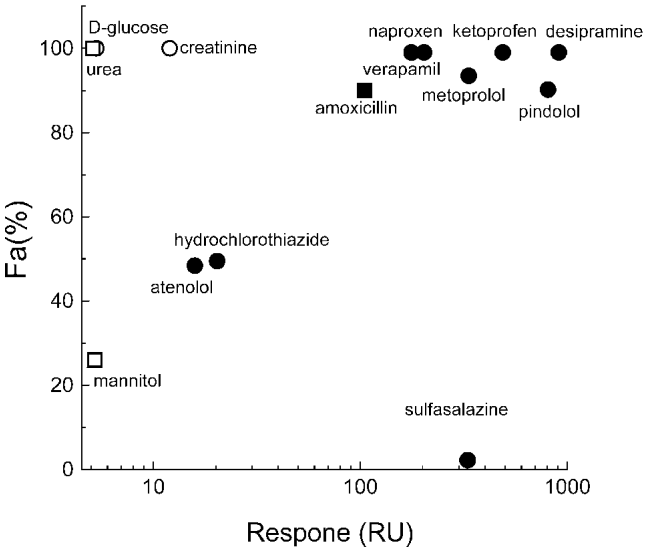

Fig. 7. Correlation between the response signal on the BBM surfaces and fraction absorbed in humans; symbols correspond to drugs classified as being absorbed via the transcellular (●), paracellar (□), active transport (○), and active transport/transcellaur (■) route.

Thus, all drugs were randomly injected, and the average response level of each drug was obtained from differently prepared BBM surfaces. Figure 5 emphasizes the reproducibility and reliability of a drug, pindolol, binding to the BBM surface. The resonance signals of 802 ± 7.3 RU for pindolol were overlaid for five injections to each randomly prepared BBM surface, and the standard deviation was less than 1%. It is suggested that the response signal of pindolol provides an independent assessment of the total experimental noise, the BBM surface preparation, and the regeneration step. As demonstrated by the small deviation between replicate binding cycles, the interaction of the drug with the BBM surface is reproducible, despite the surface being stripped and rebuilt between each drug injection.

To assay the interaction between drugs and BBM surfaces, 14 drugs were injected over the BBM surfaces. The

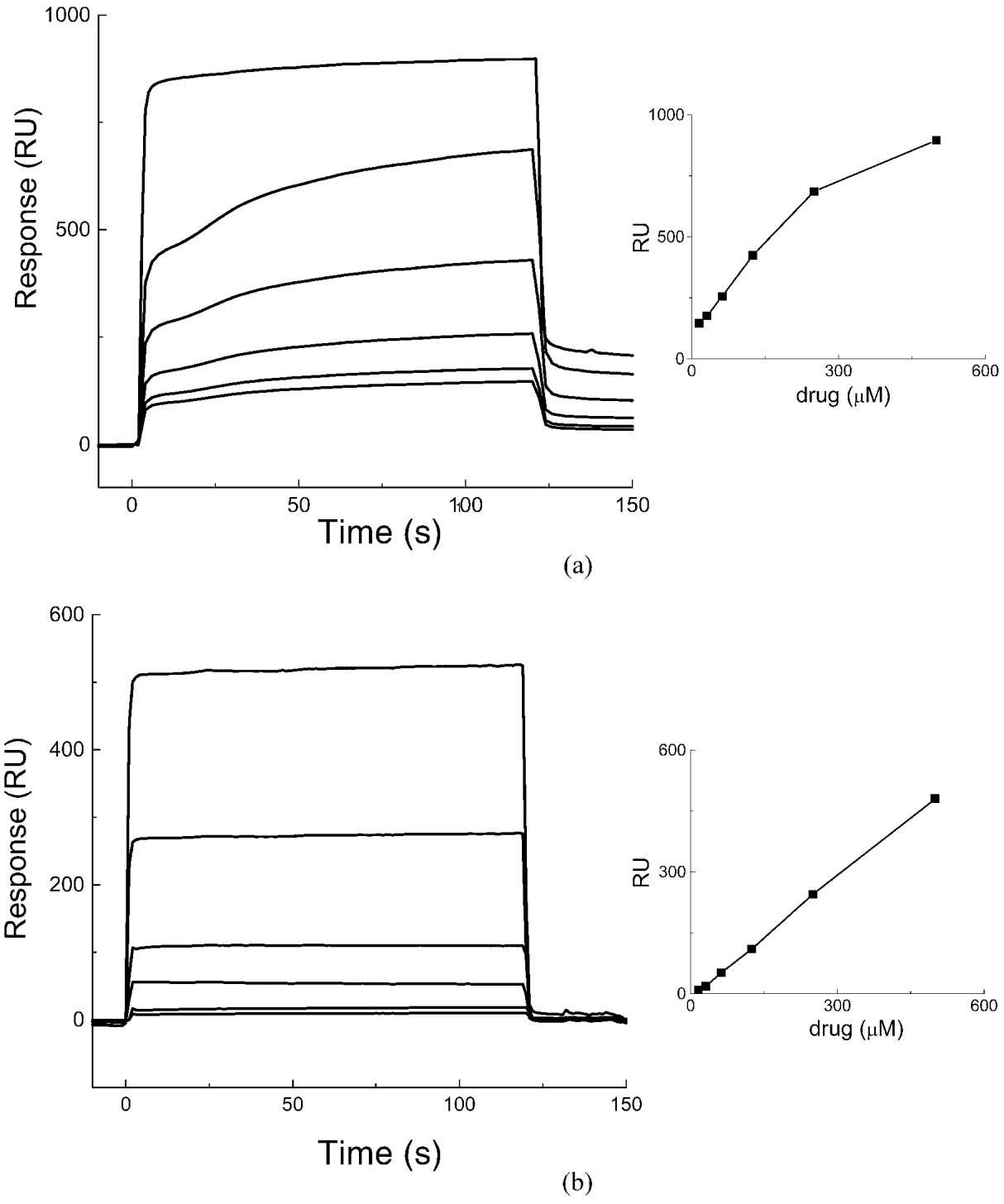

Fig. 8. The concentration dependence of the drug/BBM surface interaction was tested for (a) desipramine and (b) ketoprofen. Each drug was injected at 15.65, 31.3, 62.5, 125, and 500 μM. The inset showed the binding response at the end of the injection plotted vs. drug concentration.

drugs can be divided into three classes based on their transport mechanisms across the intestinal membrane (Table I); transcellular (t), paracellular (p), and actively transported by carrier-medicated mechanism (c). By using these diverse drug panels, we could evaluate the reliability of SPR technology to predict drug permeations in human (Fig. 6). The drugs gave a range of maximum responses during the association phase from 898 ± 23 RU for desiparamine to 5 ± 0.7 RU for mannitol. The overlay plot, showing typical sensorgrams, demonstrates that steady-state binding levels are rapidly achieved and that there is a rapid release of drugs from the BBM surfaces. Moreover, typical sensorgrams showed the degree of drug interaction to BBM surfaces. Five drugs produced visually identifiable dissociation events; the responses did not return to the baseline during washing step: (a) desipramine, (e) metoprolol, (g) verapamil, (h) amoxicillin, (i) hydrochlorothiazide. It means that these drugs have formed a very stable complex with the BBM surfaces. In contrast, the binding responses for other nine drugs immediately return to baseline, indicating an overall weaker interaction.

Figure 7 shows the correlation between the drug responses on BBM surfaces and F_a values in human. Most drugs (except sulfasalazine) absorbed via transcellular route (filled circle) show a sigmoidal relationship to human F_a values. The moderately and highly absorbed drugs are clearly separated from each other. Amoxicillin absorbed by the active transport and paracellular transport also present a reasonable binding response on the BBM surface. But sulfasalazine with a low F_a value (12%) shows a high binding response (326 ± 12 RU). It has been known that the absorption of sulfasalazine is variable *in vitro* and/or *in vivo* (19). This difference may be due to the bioavailability of sulfasalazine *in vivo* and a significant efflux of sulfasalazine (20). However, all actively transported and paracellular-mediated drugs show lower binding responses compared to human F_a values. Although the active or paracellular transport is possible, D-glucose and urea (F_a = 100%) seem to be difficult to assay with this method because of their small molecular weight (MW < 200) and low binding to hydrophobic lipid molecules in the BBM surface (5,8). But, we assumed that this seems to be a less important limitation for this drug screening method using this SPR system, as the majority of drugs have a molecular weight above 200.

To investigate further the difference in binding kinetics, concentration series of two drugs were examined. As shown in Fig. 8, all of the drugs exhibit reproducible, concentration-dependent binding responses according to drug concentrations (16.5–500 μM). In Fig. 8, ketoprofen and desipramine presented the distinctive different concentration-dependent kinetics based on their dissociation phase. After injection, ketoprofen rapidly reached an equilibrium plateau and returns quickly to baseline after the end of injection, indicating an overall weak interaction to the BBM surface. However, desipramine shows a slow increase in the response throughout the injection, and it does not return to the baseline during the dissociation phase. It indicates that desipramine has formed a very stable complex with the BBM surface. The kinetics of each drug depending on various concentrations at the end of injection is as shown in the insets in Fig. 8. As expected, the response level of ketoprofen increases linearly with the injected drug concentration. However, the curvature of desipramine strongly depends on the injected drug concentration.

CONCLUSIONS

In summary, the current study demonstrates that immobilized BBM surfaces on the L1 chip closely mimic the intact small intestine surface and can predict the interaction between drugs and BBM surfaces in one run. The immobilized BBM surface was very stable, and the BBM surface was readily regenerated by injecting a new BBM vesicle solution. The drug binding events on the BBM immobilized L1 chip directly provide important information that predicts F_a in humans for the transcellularly absorbed drugs. But, the active and paracellar-mediated transport mechanisms seem to be difficult to identify using our SPR technology. The throughput to assay each drug at a single concentration is 100 drugs for 24 h. Moreover, this SPR analysis exhibited advantages such as lack of labeling requirements, the real-time acquirement of various results, and the repeated use for various drugs.

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REFERENCES

1. P. Artursson and J. Karlsson. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophs. Res. Commun.* **175**:880–885 (1991).
2. H. Lennernäs, S. Nylander, and A. L. Ungell. Jeunal permeability: A comparision between the Ussing chamber technique and the single-pass perfusion in humans. *Pharm. Res.* **14**:667–671 (1996).
3. P. Lundahl and F. Beigi. Immobilized liposome chromatography of drugs for model analysis of drugs for model analysis of drug-membrane interactions. *Adv. Drug Deliv. Rev.* **23**:221–227 (1997).
4. K. Balon, B. U. Riebesehl, and B. W. Müller. Drug liposome partitioning as a tool for the prediction of human passive intestinal absorption. *Pham. Res.* **16**:882–888 (1996).
5. U. Hopfer, K. Nelson, J. Perrotto, and K. J. Isselbacher. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**:25–32 (1973).
6. S. Winiwarter, N. M. Bonham, F. Ax, A. Hallberg, H. Lennernäs, and A. Karlén. Correlation of human jejunal permeability (in vivo) of drugs with experimentally and theoretically derived parameters. A multivariate data analysis approach. *J. Med. Chem.* **41**:4939–4949 (1998).
7. K. Palm, P. Stenberg, K. Luthman, and P. Atrursson. Polar molecular surface properties predict the interstinal absrotion of drugs in humans. *Pharm. Res.* **14**:568–571 (1998).
8. E. Danelian, A. Karlén, R. Karlsson, S. Winiwarter, A. Hansson, S. Löfås, H. Lennernäs, and M. D. Hämäläinen. SPR biosensor studies of the direct interaction between 27 drugs and a liposome surface: Correlation with fraction absorbed in humans. *J. Med. Chem.* **43**:2083–2086 (2000).
9. D. G. Myszka and R. L. Rich. Implementing surface plasmon resonance biosensors in drug discovery. *Pharm. Sci. Technol. Today* **3**:310–317 (2000).
10. S. C. L. Baird, E. S. Courtenay, and D. G. Myszka. Surface plasmon resonance characterization of drug/liposome interactions. *Anal. Biochem.* **310**:93–99 (2002).
11. S. Cho, J. H. Park, J. Yu. Y Lee, Y. Byun, H. C. Chung, I. C. Kwon, and S. Y. Jeong. Preparation and characterization of reconstructed small intestinal brush border membranes for surface plasmon resonance analysis. *Pharm. Res.* **21**:55–60 (2004).
12. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller, and G. Semenza. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-

glucose and choline transport systems. *Biochim. Biophy. Acta* **506**:136–154 (1978).
13. S. R. A. Torre, M. A. Serrano, F. Alvarado, and J. M. Medina. Carrier-medicated L-lactate transport in brush-border membrane vesicles from rat placenta during late gestation. *Biochem. J.* **278**: 535–541 (1991).
14. K. Walter, C. Schütt, and H. U. Bergmeyer. *Methods of Enzymatic Analysis*, Academic Press, New York, 1974.
15. M. R. Webb. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc. Natl. Acad. Sci. U.S.A.* **89**:4884–4887 (1992).
16. J. Schmitz, H. Preiser, D. Maestracci, B. K. Ghosh, J. J. Cerada, and R. K. Crane. Purification of the human intestinal brush border membrane. *Biochim. Biophys. Acta* **323**:98–112 (1973).
17. E. Erb, X. Chen, S. Allen, C. J. Roberts, S. J. B. Tendler, M. C. Davies, and S. Forsén. Characterization of the surfaces generated by liposome binding to the modified dextran matrix of a surface plasmon resonance sensor chip. *Anal. Biochem.* **280**:29–35 (2000).
18. D. G. Myszka. Improving biosensor analysis. *J. Mol. Recognit.* **12**:279–284 (1999).
19. D. B. Jack. *Handbook of Clinical Pharmacokinetic Data*, Macmillan Publisher Ltd., England, 1994.
20. E. Liang and M. Yazdanian. Investigation of sulfasalazine as a substrate of efflux pumps in caco-2 cells. *Pharm. Sci* **1**:2564 (1999).