# Multiple Linear Regression Analysis Indicates Association of P-Glycoprotein Substrate or Inhibitor Character with Bitterness Intensity, Measured with a Sensor

**KENTARO YANO,[1] SUZUNE MITA,[1] KAORI MORIMOTO,[2] TAMAMI HARAGUCHI,[3] HIROSHI ARAKAWA,[1] MIYAKO YOSHIDA,[3] FUMIYOSHI YAMASHITA,[4] TAKAHIRO UCHIDA,[3] TAKUO OGIHARA[1]**

[1]Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Gunma 370-0033, Japan
[2]Department of Drug Absorption and Pharmacokinetics, Tohoku Pharmaceutical University, Sendai, Miyagi 981-8558, Japan
[3]Department of Clinical Pharmaceutics, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo 663-8179, Japan
[4]Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo, Kyoto 606-8501, Japan

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**ABSTRACT:** P-glycoprotein (P-gp) regulates absorption of many drugs in the gastrointestinal tract and their accumulation in tumor tissues, but the basis of substrate recognition by P-gp remains unclear. Bitter-tasting phenylthiocarbamide, which stimulates taste receptor 2 member 38 (T2R38), increases P-gp activity and is a substrate of P-gp. This led us to hypothesize that bitterness intensity might be a predictor of P-gp-inhibitor/substrate status. Here, we measured the bitterness intensity of a panel of P-gp substrates and nonsubstrates with various taste sensors, and used multiple linear regression analysis to examine the relationship between P-gp-inhibitor/substrate status and various physical properties, including intensity of bitter taste measured with the taste sensor. We calculated the first principal component analysis score (PC1) as the representative value of bitterness, as all taste sensor's outputs shared significant correlation. The P-gp substrates showed remarkably greater mean bitterness intensity than non-P-gp substrates. We found that $K_m$ value of P-gp substrates were correlated with molecular weight, log *P*, and PC1 value, and the coefficient of determination ($R^2$) of the linear regression equation was 0.63. This relationship might be useful as an aid to predict P-gp substrate status at an early stage of drug discovery. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** P-glycoprotein; substrate specificity; inhibition; bitterness intensity; Log P; Transporters; absorption; solubility; Multivariate analysis; Physicochemical properties; multiple linear regression analysis; correlation efficient

## INTRODUCTION

P-glycoprotein (P-gp) is an efflux transporter in small intestine,[1] liver,[2] kidney,[3] and brain,[4] serving to transport xenobiotics and/or toxic compounds out of cells, including tumor cells.[5,6] Therefore, this transporter is involved in poor gastrointestinal absorption of various drugs and multidrug resistance in tumor cells. P-gp substrates tend to have higher lipophilicity and/or larger molecular weight than nonsubstrates, but the basis of the substrate specificity of P-gp remains unclear. Nevertheless, it is very important to determine whether a compound may be a substrate of P-gp at an early stage of drug discovery, in order to predict its gastrointestinal absorption and distribution to tumor tissues.

Most compounds that are poisonous to humans have a bitter taste.[7] Bitterness is sensed by bitter taste-sensing type 2 receptors (T2Rs), which are expressed in taste buds on the tongue, palate epithelium, and mucosa of the gastrointestinal tract.[8–10] Among them, T2R member 38 (T2R38) is expressed in the gastrointestinal tract and this receptor is stimulated by phenylthiocarbamide (PTC), which is a bitter substance. Interestingly, it was reported that P-gp mRNA and transport activity were upregulated by PTC in Caco-2 cells and rat intestine. Further, this compound is a P-gp substrate.[11]

This led us to hypothesize that P-gp-inhibitor/substrate character might be associated with bitter taste. To investigate this hypothesis, we measured the bitterness intensity of P-gp substrates and non-P-gp substrates using six taste sensors, which yields values that are well correlated with those obtained in human gustatory sensation tests.[12,13] We then used multiple regression analysis to examine the contributions of various physical properties, including the first principal component ana1ysis score (PC1) as value indicating a representative character of bitterness intensity obtained by using taste sensors, to P-gp inhibition and substrate recognition. On the basis of the results, we propose an equation to predict whether compounds are P-gp substrates or non-P-gp substrates.

## METHODS

### Materials

Hanks' balanced salts, rhodamine123 (Rho123), estrone 3-sulfate sodium salt, and theophylline were purchased from Sigma–Aldrich Inc. (St. Louis, Missouri). Oseltamivir was purchased from Sequoia Research Products (Pangbourne,

**Abbreviations used:** P-gp, P-glycoprotein; T2R, taste-sensing type 2 receptor; C/M ratio, ratio of intracellular concentration to medium concentration; PCA, principal component analysis; PC1, the first PCA score; M.W., molecular weight; PTC, phenylthiocarbamide; Rho123, rhodamine123, CPA, change of membrane potential by adsorption.

*Correspondence to*: Takuo Ogihara (Telephone: +81-27-352-1180; Fax: +81-27-352-1118; E-mail: togihara@takasaki-u.ac.jp)

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UK). Colchicine, cyclosporin A, etoposide, hydrocortisone, quinidine, verapamil hydrochloride, vinblastine sulfate, 5-FU, antipyrine, caffeine, methotrexate, sodium taurocholate, pravastatine sodium salt, and quinine hydrochloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fexofenadine hydrochloride and uric acid were purchased from Tokyo Chemical Industry Company, Ltd. (Tokyo, Japan) and ChromaDex, Inc. (Irvine, California), respectively. Other reagents were of analytical grade commercial products. P-gp-overexpressing porcine kidney epithelial cell line LLC–GA5–COL150 (MDR1 gene-transfected cells) was obtained from RIKEN Cell Bank. Medium 199 (M199), penicillin, streptomycin, and trypsin ethylenediaminetetraacetic acid were purchased from Invitrogen (Carlsbad, California).

### Measurement of Bitterness Intensity Using Taste Sensors

The bitterness intensity of P-gp substrates (10 samples) and non-P-gp substrates (nine samples) was measured with several taste sensors (SA402B, Intelligent Sensor Technology), using quinine as a standard, as described previously.[14] The non-P-gp substrates included passive diffusers (antipyrine[15] and theophylline[16]) and substrates of multidrug-resistance-associated protein (estrone 3-sulfate[17]), breast cancer-resistance protein (methotrexate, pravastatin,[18] and uric acid[19]), bile salt export pump (taurocholate[20]), and an unknown transporter (not P-gp) (caffeine[21]). The detection part of the taste-sensing system consists of AN0, AC0, and BT0 sensors, which act as working electrodes and are composed of lipid/polymer membranes, and a reference electrode. Drugs with bitterness are adsorbed on the hydrophobic part of the membrane and change the membrane potential by altering the charge density.[22] Each sensor's electrodes were dipped into the reference solution (Vr) to determine the baseline, and then into the sample solution (Vs). The relative sensor output was represented as the potential difference (Vs−Vr) between the sample and the reference solution. When the electrode was immersed into the reference solution a second time, the new potential of the reference solution was defined as Vr′. The difference between the potential of the reference solution before and after sample measurement (Vr′−Vr) was defined as CPA (change of membrane potential by adsorption), and is a measure of the aftertaste of bitterness.

### Principal Component Analysis of Bitterness Intensity

Because six measures of bitterness intensity obtained from the taste sensor measurement were highly intercorrelated, principal component analysis (PCA) was carried out to condense information from the measurements. Data were standardized to a mean of zero with a standard deviation of unity, and eigenvalues and eigenvectors were then calculated for the correlation matrix. Taking the eigenvectors as loadings, the standardized data matrix was orthogonally decomposed to calculate the corresponding PCA scores. The first PCA score (PC1) was that that gave the largest eigenvalue, followed by PC2, PC3, PC4, PC5, and PC6. A series of calculations were made with the function "prcomp(PCA scores)" from the freely available statistical package R (http://cran.r-project.org/).

### Cell Culture

LLC–GA5–COL150 cells were cultured in M199 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 150 ng/mL colchicine at 37°C in an atmosphere of 5% $CO_2$ at 95% relative humidity. LLC–GA5–COL150 cells were seeded on 12-well collagen-coated plates (Becton Dickinson Bioscience, Bedford, Massachusetts) at a cell density of $3.0 \times 10^5$ cells/mL. Cells were grown for 7 days, and then used to evaluate Rho123 accumulation. The medium was changed to M199 without colchicine the day prior to the accumulation study.

### Accumulation Study

Uptake studies with LLC–GA5–COL150 cells were performed at 37°C in Hank's balanced salt solution (HBSS) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) buffer containing 10 μM Rho123 as a substrate of P-gp. Cells were preincubated with or without 100 μM of the 18 test drugs for 15 min. After preincubation, Rho123 uptake experiments were performed in HBSS–HEPES buffer containing 10 μM Rho123 in the presence of 100 μM test drugs for 10 min. Intracellular concentrations of Rho123 were measured using a WALLAC Multilabel/Luminescence Counter (PerkinElmer, Waltham, Massachusetts) at wavelengths of 485 nm (excitation) and 538 nm (emission). The ratio of intracellular concentration to medium concentration (C/M ratio) represented the coefficient of intracellular and extracellular ratio of Rho123 and was determined using the following formula: C/M ratio = intracellular concentration (μM)/extracellular concentration (10 μM)/protein concentration (mg/mL). The accumulation rates of Rho123 by cells treated with test drugs were compared with the control (Rho123 alone).

### Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Correlation analysis of six kinds of bitter taste parameter was conducted first. On the basis of the results, PC1 was selected as a representative value of bitterness, and the two-tailed *t*-test was used to compare bitterness intensity between P-gp substrates and non-P-gp substrates. For $p < 0.05$ or $p < 0.01$, differences between means were considered significant. Multiple linear regression analysis for the inhibition rates of P-gp or $K_m$ values (Michaelis constants) of P-gp for its substrates was conducted against various physical properties, including bitterness intensity. Values of physical properties and $K_m$ values of P-gp for its substrates were taken from Sci Finder, ChemDraw, or previous reports.[23–28] The $K_m$ values of non-P-gp substrates were standardized at 10,000 μM, which indicated that the concentrations of drugs were clearly nonsubstrate.[29] The relationship between dependent variable ($y$) and explanatory variable ($x$) was represented by the following equation:

$$y_i = \alpha_0 + \alpha_1 \cdot x_1 + \alpha_2 \cdot x_2 + \cdots$$

$R^2$ value is the coefficient of determination.

## RESULTS

### Measurement of Bitterness Intensity of P-gp and Non-P-gp Substrates Using Taste Sensors

To examine the idea that P-gp-inhibitor/substrate character might be associated with bitter taste, we measured the values of bitterness intensity for Rho123 (a good P-gp substrate), verapamil hydrochloride (a weak substrate), and antipyrine

(a nonsubstrate) at 100 μM, using different bitterness sensors (AN0, AC0, BT0, and each corresponding CPA value). The resulting patterns of values are shown in Figure 1. The solutions of both Rho123 (closed square) and verapamil hydrochloride (closed triangle) showed clear bitterness intensity values with all sensors, although the intensity differed from sensor to sensor. It is noteworthy that antipyrine solution (open circle) showed almost no bitterness with any of the sensors.

Next, the bitterness intensity values of P-gp substrates (10 drugs) and non-P-gp substrates (9 drugs) were evaluated with these sensors. As shown in Table 1, most P-gp substrates showed higher bitterness intensities than non-P-gp substrates. Table 2 shows the correlation coefficients among the six bitterness measurements. The values of bitterness intensity obtained with different sensors were not independent ($0.787 < r < 0.959$). Therefore, PCA was conducted to determine values indicating a representative character of bitterness intensity with each taste sensor. The contribution rate of PC1 indicated 0.90. The average PC1 value of P-gp substrates ($1.01 \pm 0.84$) was significantly higher ($^*p < 0.05$) than that of non-P-gp substrates ($-1.23 \pm 0.08$) (Fig. 2).

### Physical Properties, $K_m$ Values, and Inhibition of Rho123 Transport of P-gp and Non-P-gp Substrates

The physical properties of the compounds are summarized in Table 3. The mean molecular weight (M.W.), log *P* (an indicator of lipophilicity) and C/M ratio of P-gp substrates

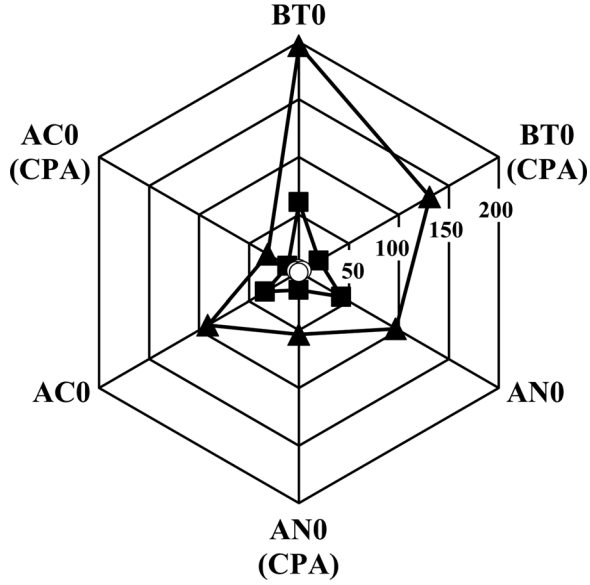


**Figure 1.** Measurement of bitterness intensity of Rho123 (a good P-gp substrate), verapamil hydrochloride (a weak substrate), and antipyrine (a nonsubstrate) using several taste sensors. Rho123 (■:closed square), verapamil hydrochloride (▲:closed triangle), and antipyrine (◯:opened circle) were dissolved in purified water at 100 μM and the bitterness intensity of the solutions was measured with various taste sensors. AN0, AC0, and BT0 are relative values of each sensor output. AN0 (CPA), AC0 (CPA), and BT0 (CPA) correspond to after taste.

**Table 1.** Measured Bitterness Intensity Values of P-gp Substrates (10 Samples) and Non-P-gp Substrates (9 Samples) Using a Taste-Sensing System with Various Sensors

| | Bitterness Intensity (mV) | | | | | |
|---|---|---|---|---|---|---|
| Drugs | BT0 | BT0 (CPA) | AN0 | AN0 (CPA) | AC0 | AC0 (CPA) |
| P-gp Substrates | | | | | | |
| Colchicine | −0.026 | −0.039 | 0.099 | −0.001 | 0.303 | −0.034 |
| Cyclosporin A | 2.449 | 3.360 | 0.485 | −0.020 | −7.413 | −0.237 |
| Etoposide | 0.922 | 1.046− | 0.511 | −0.053 | −1.972 | −0.117 |
| Fexofenadine hydrochloride | 106.783 | 33.868 | 77.278 | 27.955 | 86.330 | 26.918 |
| Hydrocortisone | 5.556 | 0.075 | 6.572 | 0.042 | 0.896 | −0.134 |
| Oseltamivir phosphate | 25.720 | 0.803 | 28.805 | 5.118 | 9.210 | 4.353 |
| Quinidine | 62.235 | 31.618 | −6.563 | 3.168 | 13.908− | 12.573 |
| Rhodamine123 | 196.515 | 131.155 | 97.105 | 53.670 | 90.873 | 31.230 |
| Verapamil hydrochloride | 60.740 | 20.238 | 42.525 | 15.428 | 33.610 | 11.178 |
| Vinblastine sulfate | 160.908 | 81.555 | 52.433 | 7.938 | 55.738 | 6.295 |
| Mean | 62.180 | 30.368 | 29.925 | 11.324 | 28.148 | 9.202 |
| Non-P-gp Substrates | | | | | | |
| 5-FU | 1.008 | 0.475 | −2.708 | −0.008 | −3.278 | 0.178 |
| Antipyrine | 2.985 | 0.383 | −2.505 | −0.025 | −3.448 | −0.005 |
| Caffeine | 4.155 | 1.134 | 0.922 | 0.021 | 0.135 | −0.812 |
| Estrone 3-sulfate sodium salt | 0.587 | 0.095 | −4.888 | 0.342 | −0.039 | 0.306 |
| Methotrexate | 17.328 | 1.809 | 0.404 | −0.010 | 2.812 | −1.675 |
| Pravastatin sodium salt | 3.387 | 0.114 | −0.162 | 0.146 | 0.941 | 0.053 |
| Sodium taurocholate | 5.722 | −0.004 | 7.320 | 0.059 | 1.454 | −0.003 |
| Theophylline | 8.677 | 12.172 | 0.517 | 0.543 | 3.728 | 8.798 |
| Uric acid | 12.823 | 0.095 | 14.542 | 0.016 | 1.745 | 0.100 |
| Mean | 6.297 | 1.808 | 1.494 | 0.121 | 0.450 | 0.771 |
| Positive Control | | | | | | |
| Quinine hydrochloride | 81.645 | 30.408 | 22.633 | 3.720 | 35.310 | 11.038 |

Nineteen drugs were dissolved in purified water at 100 μM and the bitterness intensity of the solutions was measured with various taste sensors. Data are mean values.

**Table 2.** Correlation Coefficients of Bitterness Intensity Values Obtained with Various Sensors

| *r* | BT0 | BT0 (CPA) | AN0 | AN0 (CPA) | AC0 | AC0 (CPA) |
|---|---|---|---|---|---|---|
| BT0 | | – | – | – | – | – |
| BT0 (CPA) | 0.959[a] | | – | – | – | – |
| AN0 | 0.885[a] | 0.817[a] | | – | – | – |
| AN0 (CPA) | 0.826[a] | 0.844[a] | 0.914[a] | | – | – |
| AC0 | 0.934[a] | 0.853[a] | 0.954[a] | 0.892[a] | | – |
| AC0 (CPA) | 0.826[a] | 0.787[a] | 0.851[a] | 0.905[a] | 0.917[a] | |

[a]Significant correlation between each sensor output ($p < 0.01$).

(average values: 545.78 ± 82.02 g/mol, 2.66 ± 0.47, and 131.39 ± 13.84%) were higher than those of non-P-gp substrates (296.98 ± 51.58 g/mol, 0.29 ± 0.45, and 114.34 ± 8.43%). The mean $K_m$ values of P-gp substrates were 585.75 ± 413.50 μM.

### Correlations of Physical Properties and PC1

Table 4 shows the correlations among physical properties and PC1. The correlation coefficient ($r$ value) between the PC1 value and log $P$ was 0.55, but PC1 did not correlate with M.W. ($r = 0.15$). In addition, molecular weight was rather correlated with log $P$ ($r = 0.59$).

### Multiple Linear Regression Analysis of Physical Properties Against Substrate Recognition ($K_m$ Value) and Inhibition of P-gp

Multiple linear regression analysis showed that molecular weight, log $P$, and PC1 were correlated with $K_m$ value for P-gp. The following equation was obtained:

$$y(\log K_m) = -0.00219\text{M.W.} - 0.309\log P - 0.193\text{PC1} + 4.03 \quad (1)$$

The coefficient of determination ($R^2$) of this equation was 0.63.

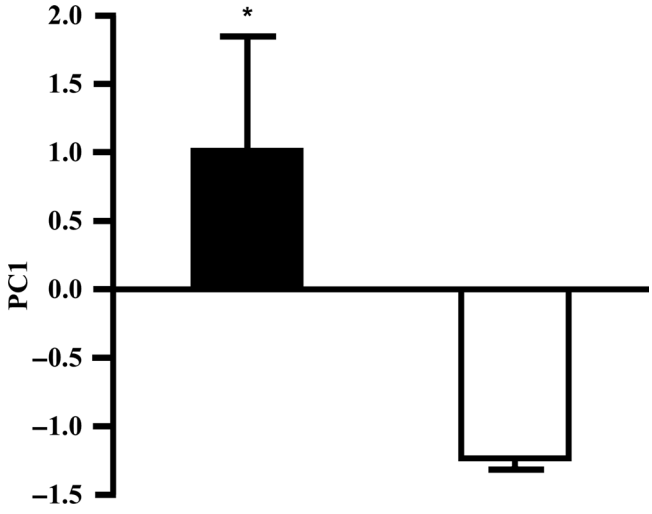


**Figure 2.** Bitterness intensity of P-gp substrates and non-P-gp substrates. The number of P-gp substrates (■) and non-P-gp substrates (□) was 10 and nine, respectively. The concentration of each drug was 100 μM. Data represents the mean ± SEM (*$p < 0.05$).

Moreover, the values of M.W., log $P$, and PC1 were also correlated with P-gp inhibition rate.

$$y(\text{inhibition rate}) = 0.0487\text{M.W.} + 12.1\log P - 6.49\text{PC1} + 80.6 \quad (2)$$

The multiple $R^2$ of this equation was 0.57.

## DISCUSSION

As shown in Table 1, the bitterness intensity of P-gp substrates was higher than that of non-P-gp substrates. The six bitterness sensor outputs for the test drugs were highly correlated with each other (Table 2). Therefore, principal components were calculated to determine the representative value of six bitter sensor outputs (Table 3). Among them, PC1 alone, as the most representative value, indicated a high proportion of variance (0.90) alone. The mean PC1 value of P-gp substrates was significantly higher than that of non-P-gp substrates, suggesting that P-gp substrates tend to have a bitter taste. However, it remains to be determined whether the substances with large PC1 scores necessarily stimulate bitter taste receptors.

Multiple linear regression analysis also indicated that $K_m$ value was correlated with M.W., log $P$, and PC1. The multiple linear regression equation Eq. (1) showed that substrates with large M.W., log $P$, and/or PC1 tended to have low $K_m$. Thus, P-gp substrate status appeared to be associated at least in part with the bitterness intensity of drugs. The regression equation for $K_m$ value had a coefficient of determination of $R^2 = 0.63$ Eq. (1). There was not a strong correlation among M.W., log $P$, and PC1 ($r < 0.7$) (Table 4). In addition, although the p$K$a values of drugs were investigated as one of their physical properties, nine of the 20 drugs were shown to be neutral substances; thus, the predictive equation including p$K$a values could not be calculated. Moreover, we used multiple linear regression analysis to examine the association of M.W., log $P$, and PC1 with P-gp inhibition rate. We found that M.W., log $P$, and PC1 were correlated with P-gp inhibition and the multiple linear regression equation Eq. (2) had substantially a predictive power ($R^2 = 0.57$). However, only M.W. and log $P$ without PC1 might be able to predict the P-gp inhibition rate, as the PC1 was negatively correlated with in Eq. (2). The compounds with high-bitterness intensity did not necessarily have the strong inhibition effect in our predictive model. Furthermore, PTC was well known as a stimulator of bitter taste receptor, T2R38, and reported as a P-gp substrate and upregulator of P-gp. However, it was unknown how large PTC's $K_m$ value was. Thus, we measured the bitterness intensity of PTC and calculated its $K_m$ value and P-gp inhibition rate. Its PC1 value was −1.14, and its $K_m$ value and inhibition rate were calculated at 5370 μM and 102.7%, respectively, using our Eqs. (1) and (2). It was suggested that PTC was a weak substrate of P-gp.

## CONCLUSIONS

Our results indicate that bitterness intensity was correlated with $K_m$ value for P-gp, suggesting that P-gp substrates tend to have high-bitterness intensity, as indicated by Eq. (1). As the bitterness intensity can be readily measured using a taste-sensing system, this relationship may be a useful tool for predicting whether compounds might be P-gp substrates or not at

**Table 3.** Physical Properties, Bitterness Intensity, and P-gp Inhibition Rate of Test Compounds and $K_m$ of P-gp Substrates

| Drugs | Molecular Weight (g/mol) | log *P* | PC1 | C/M Ratio (%) | $K_m$ (μM) |
|---|---|---|---|---|---|
| P-gp Substrates | | | | | |
| Quinine hydrochloride | 396.61 | 2.82 | 1.00 | - | 10[22] |
| Colchicine | 399.44 | 1.07 | −1.36 | 77.80 | 7.74[23] |
| Cyclosporin A | 1202.61 | 2.79 | −1.42 | 201.04 | 1.76[23] |
| Etoposide | 588.56 | 0.28 | −1.37 | 96.70 | 255[23] |
| Fexofenadine hydrochloride | 538.12 | 3.73 | 4.15 | 98.48 | 150[24] |
| Hydrocortisone | 362.46 | 1.76 | −1.22 | 100.42 | 1498[25] |
| Oseltamivir phosphate | 410.40 | 1.71 | −0.29 | 105.40 | 4500[26] |
| Quinidine | 324.42 | 2.82 | 0.20 | 149.12 | 9.93[23] |
| Rhodamine123 | 380.83 | 2.30 | 7.27 | - | 7.2[27] |
| Verapamil hydrochloride | 491.06 | 4.02 | 1.35 | 156.87 | 2.85[23] |
| Vinblastine sulfate | 909.05 | 5.92 | 2.81 | 196.64 | 0.8[22] |
| Mean ± SEM | 545.78 ± 82.02 | 2.66 ± 0.47 | 1.01 ± 0.84 | 131.39 ± 13.84 | 585.75 ± 413.50 |
| Non-P-gp Substrates | | | | | |
| 5-FU | 130.08 | −0.65 | −1.43 | 111.63 | – |
| Antipyrine | 188.23 | 0.44 | −1.43 | 101.85 | – |
| Caffeine | 194.19 | −0.63 | −1.34 | 83.15 | – |
| Estrone 3-sulfate sodium salt | 372.40 | 2.81 | −1.41 | 145.83 | – |
| Methotrexate | 454.44 | −0.45 | −1.25 | 73.28 | – |
| Pravastatin sodium salt | 447.52 | 2.21 | −1.32 | 132.82 | – |
| Sodium taurocholate | 537.63 | 0.25 | −1.20 | 144.93 | – |
| Theophylline | 180.20 | −0.28 | −0.70 | 116.48 | – |
| Uric acid | 168.11 | −1.11 | −1.04 | 119.12 | – |
| Mean ± SEM | 296.98 ± 51.58 | 0.29 ± 0.45 | −1.23 ± 0.08 | 114.34 ± 8.43 | |

The values of molecular weight (M.W.), log *P*, and $K_m$ were taken from several sources (see text). The inhibition rate of P-gp was represented by the C/M ratio of Rho123 (% of control) in LLC–GA5–COL150 cells.

**Table 4.** Correlation Coefficients of Physical Properties and Bitterness Intensity (PC1 Value)

| *r* | Molecular Weight (g/mol) | log *P* | PC1 |
|---|---|---|---|
| Molecular weight (g/mol) | | – | – |
| log *P* | 0.592 | | – |
| PC1 | 0.153 | 0.548 | |

an early stage of drug discovery without extensive *in vitro* or *in vivo* studies.

Other reports have attempted to identify P-gp substrates or inhibitors based on molecular similarity,[30] and other methods of classification.[31] The present results suggest that bitterness measurement will complement these approaches. Further evaluation with larger numbers of compounds seems warranted, together with an examination of the possible physical basis for the relationship.

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