REFERENCES

Thai

สมภพ ประธานธุรารักษ์ . "Herbal medicine" ใน ธานี เมฆะสุวรรณดิษฐ์, ปรีชา มนทกานติกุล, จุฑามณี สุทธิสีสังข์ และ สุรเกียรติ อาชานุภาพ, บรรณาธิการ. ตำราเภสัชบำบัด. กรุงเทพฯ: โฮลิสติก พับลิชชิ่ง, 2546. 383-402.

English

Allera, A.; Lo, S.; King, I.; Steglich, F.; and Klingmuller, D. Impact of androgenic/antiandrogenic compounds (AAC) on human sex steroid metabolizing key enzymes. Toxicology 205 (2004): 75-85.

Andrea, V. D.; Perez, L. M.; and Pozzi, E. J. S. Inhibition of rat liver UDP-glucuronosyltransferase by silymarin and the metabolite silibinin-glucuronide. Life Sciences 77 (2005): 683-692.

Babu, T. D.; Kuttan, G.; and Padikkala J. Cytotoxic and anti-tumour properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. Journal of Ethnopharmacology 48 (1995): 53-57.

Baijal, P. K.; Fitzpatrickt, D. W.; and Bird, R. P. Phenobarbital and 3-Methylcholanthrene Treatment Alters Phase I and II Enzymes and the sensitivity of the rat colon to the carcinogenic activity of azoxymethane. Food and Chemical Toxicology 35 (1997): 789-798.

Benson, A. M.; Hunkeler, M. J.; and Talalay, P. Increase of NAD(P)H:quinone reductase by dietary antioxidants: Possible role in protection against carcinogenesis and toxicity. Biochemistry 77 (1980): 5216-5220.

Bock, K. W.; Burchell, B.; Dutton, G. J.; Hanninen, O.; Mulder, G.; Owens, I. S.; Siest, G.; and Tephly, T. R. UDG-Glucuronosyltransferase activities. Biochemical Pharmacology 32 (1998): 953-955.

Bunpo, P.; Kataoka, K.; Arimochi, H.; Nakayama, H.; Kuwahara, T.; Bando, Y.;Izumi, K.; Vinitketkumnuen,Y.; and Ohnishi, Y. Inhibitory effects of *Centella asiatica* on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats. Food and Chemical Toxicology 42 (2004): 1987-1997.

Chang, T. K. H.; Chen, J.; and Yeung, E. Y. H. Effect of *Ginkgo biloba* extract on procarcinogen bioactivating human CYP1 enzymes: Identification of

isorhamnetin, kaempferol, and quercetin as potent inhibitors of CYP1B1. Toxicology and Applied Pharmacology 213 (2006): 18-26.

Cheng, C. L.; and Koo, M. W. L. Effect of *Centella asiatica* on ethanol induced gastric mucosa lesions in rats. Life sciences 67 (2000): 2647-3653.

Cheng, C. L.; Guo, J. S.; Luk, J.; and Koo, M. W. L. The healing effect of Centella extract and asiaticoside on acetic acid induced gastric ulcerinrats. Life Sciences 74 (2004): 2237-2249.

Chen S.; Wu, K.; and Knox, R. Stucture-function studies of DT-diaphorase (NQO1) and NRH quinine oxidoreductase (NQO2). Free Radical Biology & Medicine 29 (2000): 276–284.

Coleman, M. D. Human Drug Detabolism: an Introduction. Chichester: John Wiley, 2005.

Collier, A. C.; Tingle, M. D.; Keelan J. A.; Paxton, J. W.; and Mitchell M. D. A highly Sensitive fluorescent microplate method for fetermination of UDP-Glucuronosyl transferase activity in tissue and plancental cell lines. Drug Metabolism and Disposition 28 (2000): 1184-1186.

Dinkovo-Kostova, A. T.; and Talalay, P. Persuasive evidence that Quinone reductase type I (DT Diaphorase) protect cell against the toxicity of electrophiles and reactive forms of oxygens. Free Radical Biology & Medicine 29 (2000): 231-240.

Dangles, O., and Dufour, C. Flavonoids Chemistry, Biochemistry and Applications. Boca Raton: Taylor & Francis Group, 2006.

Elbakai, R. H.; Duke, J.; and El-Kadi, A. O. S. Ascorbic acid differentially modulates the induction of heme xygenase-1, NAD(P)H: quinine oxidoreductase 1 and glutathione S-transferase Ya by As^{3+} , CD^{2+} and Cr^{6+}. Cancer Letters 246 (2007): 54-62.

Ernster, L.; Ljunggren, M.; and Danielson, L. Purification and Some properties of A Highly Dicumarol-Sensitive Liver Diaphorase. Biochemical and Biophysical Research Communications 1 (1960): 88-92.

Ernster, L. DT diaphorase. Methods in Enzymology Volum X, Academic Press, NY, (1967): 309-317.

Ernster, L. DT diaphorase. Methods in Enzymology 186 (1990): 287-301.

Frame L. T.; Ozawa S; Nowell S. A.; Chou H. C.; DeLongchamp R. R.; Doerge D. R.; Lang N. P.; and Kadlubar F. F. A simple colorimetric assay for phenotyping the major human thermostable phenol sulfotransferase (SULT1A1) using platelet cytosols. Drug Metabolism and Disposition 28 (2000): 1063-1068.

Ganzera, M.; Schneider, P.; And Stupper, H. Inhibitory effects of the essential oil of chamomile (Matricaria recutita L.) and its major constituents on human cytochrome P450 enzymes. Life Sciences 78 (2006): 856-861.

Gibson, G. G. and Skett, P. Introduction to drug metabolism. New York: Chapman and Hall, 1986.

Gnanapragasama, A.; Ebenezara, K. K.; Sathisha, V.; Govindarajub, P.; and Devakia, T. Protective effect of *Centella asiatica* on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. Life Sciences 76 (2004): 585-597.

Guengerich F. P. Cytochrome P450: Structure, Mechanism, and Biochemistry. New Yok: kluwer Academic/Plenum Publisher, 2005.

Gupta, Y. K.; Kumar, M. H.; and Srivastava, A. K. effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacology, Biochemistry and Behavior 74 (2003): 579-585.

Gyamfi, M. A.; Ohtani, I. I.; Shinno, E.; and Aniya, Y. Inhibition of glutathione *S*-transferases by thonningianin A, isolated from the African medicinal herb, Thonningia sanguinea, invitro. Food and Chemical Toxicology 42 (2004): 1401-1408.

Habig, W. H.; Pabst, M. J.; and Jakoby, W. B. Glutathione *S*-Transferases. Journal Biochemical Chemistry 249 (1974): 7130-7139.

Hamid, A. A.; Shah, Z. Md.; Muse, R.; and Mohamed. Characterisation of antioxidative activities of various extracts of *Centella asiatica* (L) Urban. Food Chemistry 77 (2002): 465-469.

Hodgson, E.; and Rose, R. L. The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals. Pharmacology & Therapeutics 113 (2007): 420-428.

Horn, T. L.; Longa, L.; Cwika, M. J.; Morrissey, R. T.; Kapetanovic, I. M.; and McCormock, D. L. Modulation of hepatic and renal drug metabolizing

enzymes activities in rats by subchronic administration of farnesol. Chemico-Biological Interactions 152 (2005): 79-99.

Hussin, M.; Abdul-Hamid. A.; Mohamad. S.; Saari. N.; Ismail, M.; and Bejo, M. H. Protective effect of *Centella asiatica* extract and powder on oxidative stress in rats. Food chemistry 100 (2007): 535-541.

Inamdar, P. K.; Yeole, R. D.; Ghogare, A. B.; and Souza, N. J. D. Determination of biologically active constituents in *Centella asiatica*. Journal of Chromatography A 742 (1996): 127-130.

Ioannides C., ed. Cytochromes P450 metabolic and toxicological aspects. Florida: CRC Press, 1996.

Iqbal, M.; and Okada, S. Introduction of NAD(P)H quinine reductase by probucol: A possible mechanism for protection against chemical constituents and toxicity. Pharmacology and Toxicology 93 (2003): 259-263.

Iwasaki, K.; Tokuma, Y.; Noda, K.; and Noguchi, H. Age- and sex-related changes of sulfotransferase activities in the rat. Chemico-Biological Interactions 92 (1994): 209-217.

Iwata, N.; Minegishi, K. I.; Suzuki, K.; and Ohono, Y. An unusual profile of musk xylene-induced drug-metabolizing enzymes in rat liver. Biochemical Pharmacology 45 (1993): 1659-1665.

Jakoby, W.B. Glutathione *S*-Transferase: An Overview. Methods in Enzymology 113(1985): 496-501.

James, M. O. Increased toxicity of benzo(*a*)pyrene-7, 8-dihydrodiol in the presence of polychlorobiphenyylols. Marine Environmental Research 58 (2004): 343-346.

Jame, M. O.; Sacco, J.; and Fauz, L. R. Effects of food natural products on the biotransformation of PCBs. Environmental, Toxicology and Pharmacology 25 (2008): 211-217.

Jayashree, G.; Muraleedhara, G. K.; Sudarslal, S.; and Jacob, V. B. Anti-oxidant activity of Centella asiatica on lymphoma-bearing mice. Fitoterapia 74 (2003): 431-434.

Jayathirtha, M. G.; and Mishra, S. H. Preliminary immunomodulatory activities of methanol extracts of *Eclipta alba* and *Centella asiatica* Phytomedicine 11 (2004): 361–365.

Jorge, O. A.; and Jorge, A. D. Hepatotoxicity associated with the ingestion of *Centella asiatica*. Revista Espanola de Enfermedades 97 (2005): 115-124.

Korhonen, L. E.; Turpeinen, M.; Rahnasto, M.; Wittekindt, C.; Poso, A.; Pelkonen, O.; Raunio, H.; and Juvonen, R.O. New potent and selective cytochrome P450 2B6 (CYP2B6) inhibitors based on three-dimensional quantitative structure-activity relationship (3D-QSAR) analysis. British Journal of Pharmacology 150 (2007): 932–942.

Krippendorff, B. F.; Lienau, P.; Reichel, A.; and Huisinga, W. Optimizing classification of drug-drug Interaction potential for CYP450 iso-enzymes inhibition assays in early drug discovery. Published in Journal of Biomolecular Screening Online Nov 27, 2006.

Kornphimol Kulthong. Subchronic effectd the standard extract of Centella asiatica on rat hepatic cytochrome P450. Master's Thesis, Program in Pharmacology (Interdisciplinary Program), Graduate School, Chulalongkorn University, 2007.

Kumar M. H. V.; and Gupta Y. K. Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. Journal of Ethnopharmacology 79 (2002): 253-260.

Lin, J. H.; and Lu, A. Y. H. Inhibition and induction of cytochrome P450 and the clinical implications. Clinical Pharmacokinetic 35 (1998): 301-390.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; and Randall, R. J. Protein measurement with the foline phenol reagent. Journal of Biological Chemistry 193 (1951): 265-275.

Lyon, E. S.; Marcus, C. J.; Wang, J. L.; and Jakoby, Hydroxysteroid sulfotransferase. Methods in Enzymology 77 (1981): 197-206.

Manzi, S. F.; and Shannon, M. Drug interactions-a review. Clinical Pediatric Emergency Medicine. 6 (2005): 93-102.

Marks, B. D.; and Larson, B. R. Miniaturization and automation of cytochrome P450 inhibition assays[online]. 2008. Available from: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Drug-Discovery/DD-Misc/Drug-Discovery-Posters.html [2008, June 19]

Marks, B. D.; Smith, R. W.; Braun, H. A.; Goossens, T. A.; Christenson, M.; Ozers, M. S.; Lebakken, C. S.; and Trubetskoy, O. V. A High Throughput Screening

Assay to Screen for CYP2E1 Metabolism and Inhibition Using a Fluorogenic Vivid® P450 Substrate. Assay and Drug Development Technologies 1 (2002): 73-81.

Moon, Y. J.; Wang, X.; and Morris, M. E. Dietary flavonoids: Effects on xenobiotic and carconigen metabolism. Toxicology in Vitro 20 (2006): 187-210.

Mulder, G. J.; Hinson, J. A.; and Gillette, J. R. Conversion of the *N*-*O*-glucuronide and *N*-*O*-sulfate conjugates of *N*-hydroxyphenacetin to reactive intermediates. Biochemical Pharmacology 26 (1977): 189-196.

Parkinson, A. Casarett&Doull's: Toxicology: The Basic Science of Poisons. 6th ed. New tork: McGraw-Hill, 2001.

Punturee, K.; Wild, C. P.; and Vinitketkumneun, C. Thai medicinal plants modulate nitric oxide and tumor necrosis factor-α in J774.2 mouse macrophages. Journal of Ethnopharmacology 95 (2004): 183-189.

Randriamampionona, D.; Diallo, B.; Rakotoniriana F.; Rabemanantsoa C.; Cheuk K., Corbisier A, Mahillon J.; Ratsimamanga S.; and Jaziri M.E. Comparative analysis of active constituents in *Centella asiatica* samples from Madagascar: Application for ex situ conservation and clonal propagation. Fitoterpia 78 (2007): 482-489.

Rao, S. B.; Chetana, M.; and Devi, P. U. *Centell asiatica* treatment during postnatal period enhances learning and memery in mice. Physiology & Behavior 86 (2005): 449-457.

Rao, K. G. M.; Rao, S. M.; and Rao, S. G. *Centella asiatica* (L.) Leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats. Evidence-based Complementary and Alternative Medicine 3(3) (2006): 349-357.

Sakai-Kato, K.; Kato, M.; and Toyooka, T. Screening of inhibition of uridine diphosphate glucuronosyltransferase with a miniaturized on-line drug-metabolism system. Journal of Chromatography A 1051 (2004): 261-266.

Schultz, M.; Dutta, S.; and Tew, K. D. Inhibitors of glutathione *S*-transferase as therapeutic agents. Advanced Drug Delivery Review 26 (1997): 91-104.

Shetty, B. S.; Udupa, S. L.; Udupa, A. L.; and Somavaii, S. N. Effect of *Centella asiatica* L (Umbelliferae) on normal and dexamethasone-suppressed wound healing in Wistar Albino rats. Lower Extremity Wounds 5 (2006): 137-143.

Sheweita, S. A.; Ei-Gabar, M. A.; and Bastawy, M. Carbon tetrachloride-induced changes in the activity of phase II drug-metabolizing enzyme in the liver of male rats: role of antioxidants. Toxicology 165 (2001): 217-224.

Shinomol, G.; and Muralidahara. Effect of *Centella asiatica* leaf powder on oxidative markers in brain regions of prepubertal mice in vivo and its in vitro efficacy to ameliorate 3-NPA-induced oxidative stress in mitochondria. Phytomedicine (2008), doi:10.1016/j.phymed.2008.04.010

Shukla, A.; Rasil, A. M.; Jain, G. K.; Shankar, R.; Kulshrestha, D. K.; and Dhawan. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. Journal of Ethnopharmacology 65 (1999): 1-11.

Siddique, Y. H.; Ara, G.; Beg, T.; Faisal, M.; Ahmad, M.; Afzal, M. Antigenotoxic role of *Centella asiatica* L. extract againstcyproterone acetate induced genotoxic damage in cultured human lymphocytes. Toxicology in Vitro 22 (2008): 10-17.

Singh, P.; and Singh, S. J. Recruitment and competitive interaction between ramets and seedlings in a perennial medicinal herb, *Centella asiatica*. Basic and Applied Ecology 3 (2002): 65-76.

Soucek, P.; and Gut, I. Cytochrome P450 in rats: structures, functions, properties and revelant human forms. Xenobiotica 22 (1992): 83-103.

Surh, Y. J.; Liem, A.; Miller E. C.; and Miller J. A. Age-and sex-related differences in activation of the carcinogen 7-hydroxymethyl-12-methylbenz[a]anthracene to an electrophillic sulfuric acid ester metabolite in rats: possible involvement of hydroxysteroid sulfotransferase activity. Biochemical Pharmmology 41 (1991): 213-221.

Turpeinen, M.; Tolonen, A.; Unsitalo, J.; Jalonen, J.; Pelkonen, O.; and Laine, K. Effect of clopidogrel and ticopidine on cytochrome P450 2B6 activity as measured by bupropion hydroxylation. Clinical Pharmacological Therapy 77 (2005): 553-559.

Turella, P.; Cerella, C. ; Filomeni, G. ; Bullo, A.; Maria, F. D.; Ghibelli, L.; Ciriolo, M. R.; Cianfriglia, M.; Mattei, M.; Federici, G.; Ricci, G.; and Caccuri, A. M. Proapoptotic activity of new glutathione *S*-transferase inhibitors. American Association for Cancer Research 65 (2005): 3751-3761.

U.S. FDA. Guidance for Industry: Drug Interaction Studies-Study Design, Data Analysis, and Implications for Dosing and Labeling[online]. 2006. Available from: http://www.fda.gov/cder/Guidance/6695dft.htm[20008, October 1]

Wallin, A.; Gerdes, R. G. ; Morgenstern, R.; Jones, T. W.; and Ormstad, K. Features of microsomal and cytosolic glutathione conjugation of hexachlorobutadiene in rat liver. Chemico-Biological Interactions 68 (1988): 1-11.

Wang, L. Q.; Lehmler, H. J.; Robertson, L. W.; and James, M. O. Polychloro-biphenylols are selective inhibitors of human phenol sulfotransferase *1A1* with 4-nitrophenol as a substrate. Chemico-Biological Interactions 159 (2006): 235-246.

Wang, X. S.; Dong, Q.; Zuo, J. P.; and Fang, J. N. Structure and potential immunological activity of a pectin from *Centella asiatica* (L.) Urban. Carbohydrate Research 338 (2003): 2393-2402.

Wang, X. S.; Liu, L.; and Fang, J. N. Immunological activities and structure of pectin from *Centella asiatica*. Carbohydrate Polymers 60 (2005): 95-101.

Wattanathorn, J.; Mator, L.; Muchimapura, S.; Tongun, T.; Pasuriwong, O.; Piyawatkul, N.; Yimtae, K.; Sripanidkulchai, B.; and Singkhoraard, J. Positive modulation of cognition and mood in the healthy elderly volunteer following the administration of *Centella asiatica.* Journal of Ethnopharmacology 116 (2008): 325-332.

Weisburger, J. H.; and Chung, F. L. Mechanisms of chronic disease causation by nutritional factors and tobacco products and their prevention by tea polyphenols. Food and Chemical Toxicology 40 (2002): 1145-1154.

WHO. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. Geneva: World Health Organization, 2001.

Zainol, M. K.; Hamid, A. A; Yusof, S.; and Muse, R. Antioxidative activity and total phenolic compound of leaf, root and petiole of four accessions of *Centella asiatica* (L.) Urban. Food Chemistry 81 (2003): 575-581.

Zhang, D., Zhu, M., and Humphreys, W. G., eds. Drug Metabolism in Drug Design and Development. New Jersey: Jhon Wiley & Sons. 2007.

Appendices

Appendix A

- Verification of methods for the determination of inhibitory effect of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 activities
- Verification of methods for the determination of UDPGT, SULT, GST and NQOR activities

Table A1 Inhibitory effect of α-naphthoflavone on CYP1A2 activity

Concentration of α-naphthoflavone in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.005	9.03	9.22	9.13
0.012	33.23	34.37	34.37
0.480	80.78	83.96	83.96
1.200	87.93	90.43	90.43
3.000	90.63	91.52	91.52

Table A2 Inhibitory effect of miconazole on CYP2B6 activity

Concentration of miconazole in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.05	23.08	17.87	20.47
0.50	49.09	45.18	47.14
1.25	53.91	48.01	50.96
5.00	75.11	74.61	74.86
20.00	79.85	79.57	79.71

Table A3 Inhibitory effect of sulfaphenazole on CYP2C9 activity

Concentration of sulfaphenazole in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.04	16.40	13.98	15.19
0.10	25.21	24.99	25.10
0.26	34.94	38.54	36.74
0.64	41.69	46.86	44.27
1.60	55.00	58.64	56.82
4.00	63.86	59.45	61.65

Table A4 Inhibitory effect of miconazole on CYP2C19 activity

Concentration of miconazole in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.002	6.76	7.38	7.07
0.007	13.34	10.88	12.11
0.117	60.12	59.25	59.68
0.469	75.71	75.24	75.47
1.875	99.98	98.27	99.13

Table A5 Inhibitory effect of miconazole on CYP2D6 activity

Concentration of miconazole in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.31	3.38	1.33	2.35
0.77	21.23	23.55	21.23
1.92	53.31	55.35	54.33
4.80	72.17	76.42	74.30
12.00	94.30	98.13	96.22
30.00	96.36	100.32	98.34

Table A6 Inhibitory effect of imipramine on CYP2E1 activity

Concentration of imipramine in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
78.13	26.07	23.54	24.80
156.25	31.22	37.52	34.37
312.50	47.32	42.30	44.81
625.00	69.12	60.81	64.96
1250.00	67.96	76.49	72.22

Table A7 Inhibitory effect of ketoconazole on CYP3A4 activity

Concentration of ketoconazole in the reaction mixture (μM)	% Inhibition		
	1	2	Mean
0.04	30.06	28.14	29.10
0.10	56.42	55.31	55.87
0.64	75.69	74.66	75.17
1.60	91.47	89.96	90.71
4.00	94.90	94.29	94.59

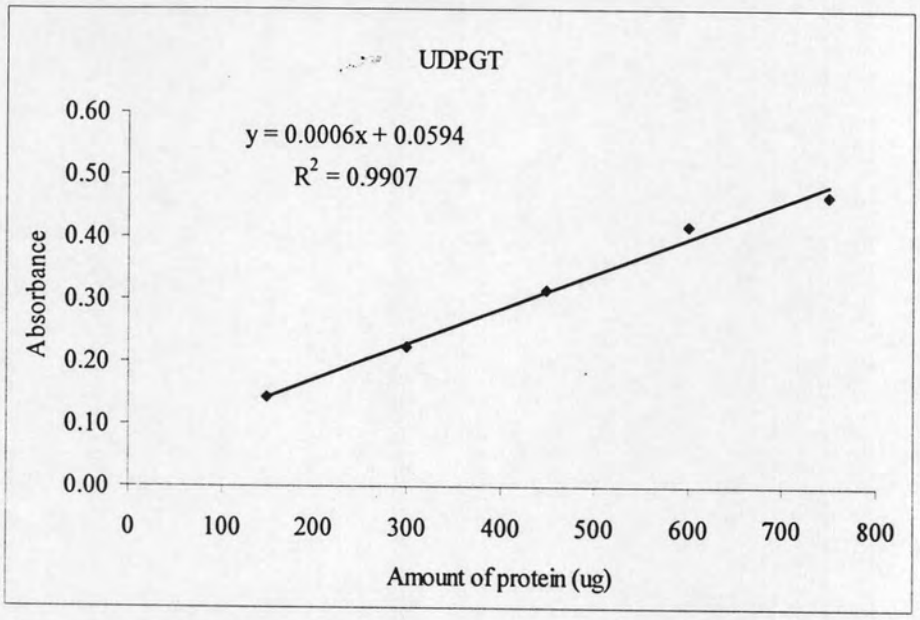

Figure A1 Linearity of the method for determination of UDPGT activity. The line was acquired by plotting the amounts of microsomal protein (in the ranges of 150-750 µg/0.5 ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9907. Each point represented the mean of duplicated reactions.

Table A8 Linearity of the method for determination of UDPGT activity in rats

Microsomal protein (µg)	absorbance
150	0.143
300	0.223
450	0.315
600	0.419
750	0.468

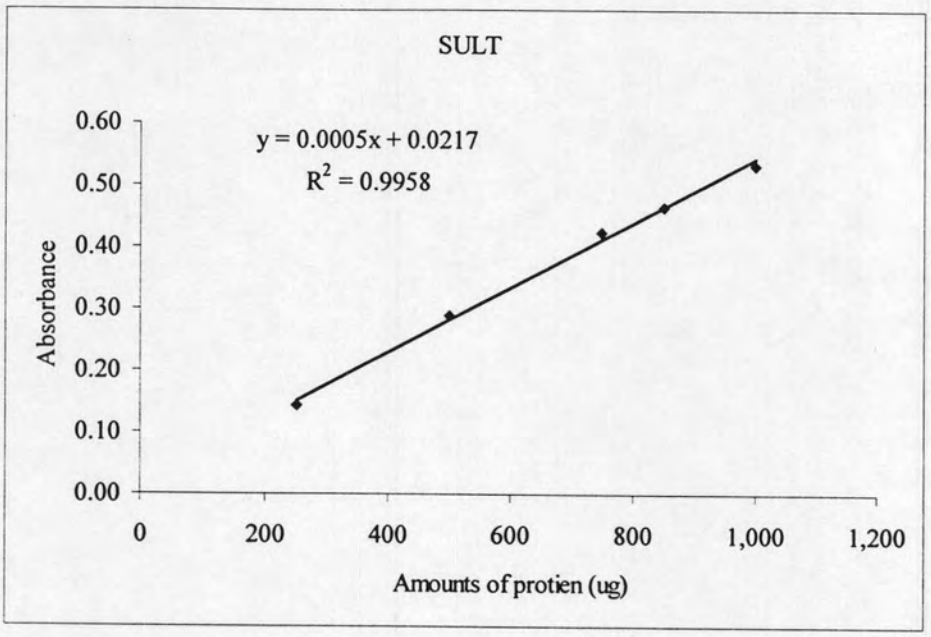

Figure A2 Linearity of the method for determination of SULT activity. The line was acquired by plotting the amounts of microsomal protein (in the ranges of 250-1,000 μg/1.5 ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9958. Each point represented the mean of duplicated reactions.

Table A9 Linearity of the method for determination of SULT activity in rats

Cytosolic protein (μg)	absorbance
250	0.143
500	0.289
750	0.426
850	0.464
1,000	0.532

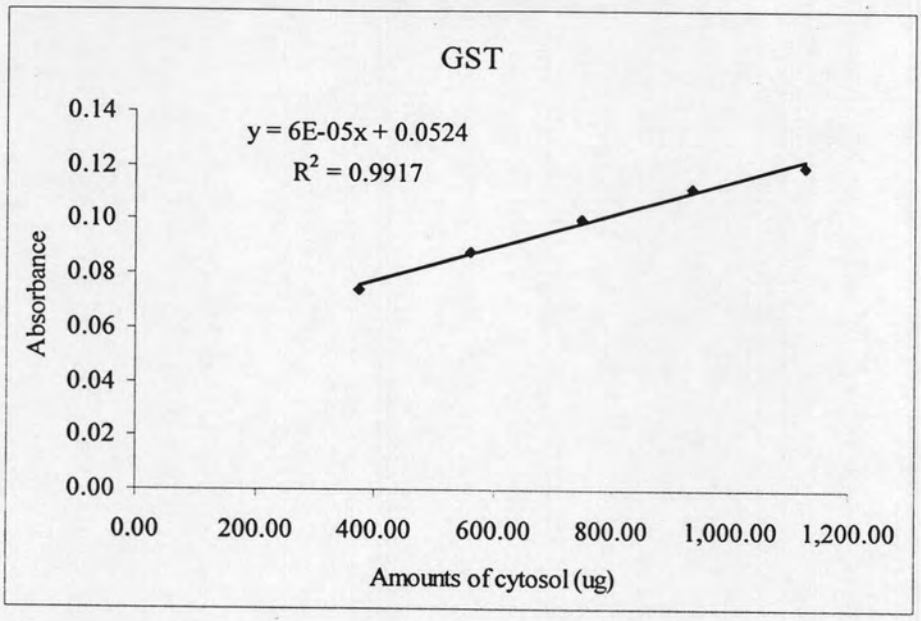

Figure A3 Linearity of the method for determination of GST activity. The line was acquired by plotting the amounts of cytosol (in the ranges of 375-1,125 μg/ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9917. Each point represented the mean of duplicated reactions.

Table A10 Linearity of the method for determination of GST activity in rats

Cytosolic protein (μg)	absorbance
375.0	0.074
562.5	0.088
750.0	0.100
937.5	0.112
1,125.0	0.120

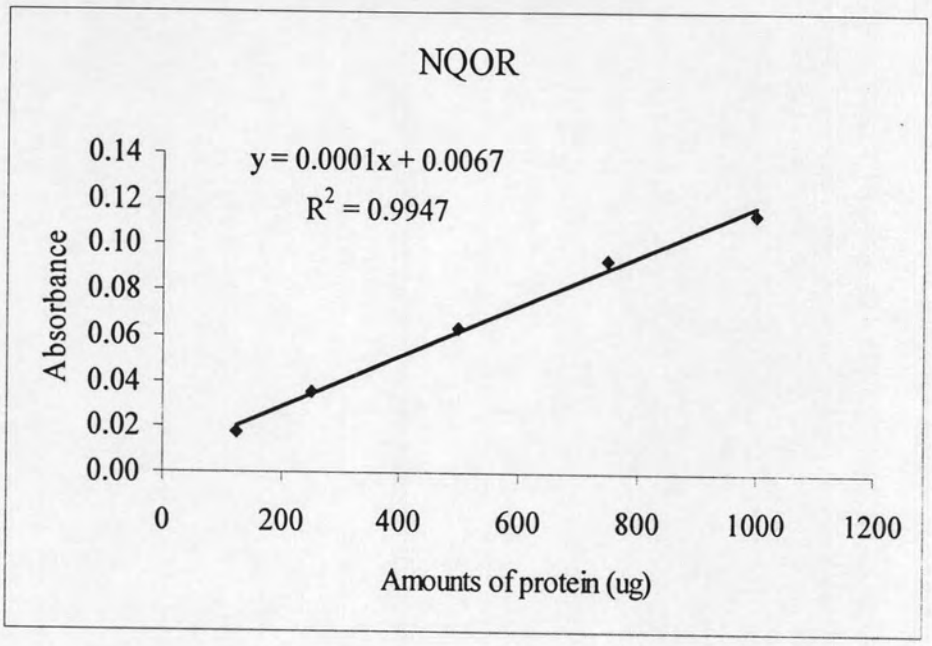

Figure A4 Linearity of the method for determination of GST activity. The line was acquired by plotting the amounts of cytosol (in the ranges of 125-1,000 µg/3 ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9947. Each point represented the mean of duplicated reactions.

Table A11 Linearity of the method for determination of NQOR activity in rats

Cytosolic protein (µg)	absorbance
125	0.018
250	0.035
500	0.063
750	0.093
1,000	0.114

Appendix B

Enzyme activity study

Table B1 Inhibitory effect of ECa 23 on CYP1A2 activity

Concentration of ECa 233 in the reaction mixture (μg/ml)	% Inhibition			
	1	2	3	Mean ± SD
125.0	15.37	16.26	9.96	13.86 ± 1.53
250.0	11.60	6.91	11.53	10.01 ± 1.51
500.0	15.27	15.56	18.09	16.31 ± 2.83
750.0	23.38	26.42	22.80	24.20 ± 3.41
1,000.0	34.20	37.76	35.17	35.71 ± 2.68

Table B2 Inhibitory effect of ECa 233 on CYP2B6 activity

Concentration of ECa 233 in the reaction mixture (μg/ml)	% Inhibition			
	1	2	3	Mean ± SD
250.0	28.21	27.79	27.05	27.68 ± 0.59
500.0	37.80	37.99	37.31	37.70 ± 0.35
750.0	52.71	46.65	52.95	50.77 ± 3.57
1,000.0	52.74	49.83	53.23	51.94 ± 1.84
2,000.0	64.36	64.57	64.50	64.47 ± 0.11

Table B3 Inhibitory effect of ECa 233 on CYP2C9 activity

Concentration of ECa 233 in the reaction mixture (µg/ml)	% Inhibition			
	1	2	3	Mean ± SD
125.0	0.00	0.00	0.00	0.00
250.0	0.00	0.13	0.00	0.04 ± 0.07
300.0	0.00	0.00	0.00	0.00
375.0	0.00	0.00	0.59	0.20 ± 0.34
500.0	0.00	0.00	0.00	0.00
600.0	0.00	0.96	0.00	0.32 ± 0.55
750.0	5.57	3.46	3.30	4.11 ± 1.27

Table B4 Inhibitory effect of ECa 233 on CYP2C19 activity

Concentration of ECa 233 in the reaction mixture (µg/ml)	% Inhibition			
	1	2	3	Mean ± SD
62.5	8.99	8.44	8.53	8.65 ± 0.29
125.0	19.73	26.96	-	23.34
250.0	35.89	45.04	35.97	38.97 ± 5.26
375.0	47.46	47.88	48.01	47.79 ± 0.29
500.0	60.93	61.22	62.35	61.50 ± 0.75
600.0	62.31	61.35	62.38	62.01 ± 0.58
750.0	67.23	66.92	67.35	67.17 ± 0.22
1,000.0	81.96	81.82	82.70	82.16 ± 0.48

(-) mean missing data

Table B5 Inhibitory effect of ECa 233 on CYP2D6 activity

Concentration of ECa 233 in the reaction mixture (μg/ml)	% Inhibition			
	1	2	3	Mean ± SD
62.5	0.00	0.00	0.00	0.00
125.0	0.00	0.00	0.00	0.00
250.0	0.00	0.00	0.00	0.00
375.0	0.59	0.00	0.00	0.20 ± 0.34
500.0	5.97	0.55	0.00	2.17 ± 3.30
600.0	14.61	15.95	11.59	14.05 ± 2.23
750.0	19.55	15.83	14.17	16.52 ± 2.76
1,000.0	38.13	38.64	32.66	36.48 ± 3.32

Table B6 Inhibitory effect of ECa 233 on CYP2E1 activity

Concentration of ECa 233 in the reaction mixture (μg/ml)	% Inhibition			
	1	2	3	Mean ± SD
25.0	0.00	0.00	0.00	0.00
37.5	0.00	0.00	0.00	0.00
50.0	0.00	0.00	0.00	0.00
75.0	0.00	0.00	0.00	0.00
100.0	0.00	0.00	0.00	0.00
150.0	0.00	0.00	0.00	0.00
200.0	0.00	0.00	0.00	0.00
250.0	0.00	0.00	0.00	0.00

Table B7 Inhibitory effect of ECa 233 on CYP3A4 activity

Concentration of ECa 233 in the reaction mixture (µg/ml)	% Inhibition			
	1	2	3	Mean ± SD
62.5	-	25.44	28.98	27.21
250.0	-	43.19	53.14	48.17
300.0	-	47.83	54.37	51.10
375.0	64.71	65.40	67.65	65.92 + 1.54
500.0	71.45	73.39	73.11	72.65 + 1.05
600.0	75.39	71.75	74.49	73.88 + 1.89
750.0	80.18	80.76	78.54	79.83 + 1.15

(-) mean missing data

Table B8 Liver microsomal protein concentration of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	57.18	71.00	86.29	107.14
2	99.00	89.94	42.11	58.29
3	94.37	86.94	100.23	83.23
4	65.56	44.67	90.94	47.00
5	34.09	56.44	91.94	79.66
6	79.94	55.62	56.23	50.11
7	55.51	76.94	61.17	82.66
8	65.22	76.22	71.33	65.07
9	72.67	64.56	60.18	56.89
10	51.89	55.07	65.50	66.00

Unit expressed as mg/ml

Table B9 Liver microsomal protein concentration of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	23.43	27.43	40.00	74.86
2	33.43	43.29	58.00	72.72
3	45.14	28.80	41.37	48.80
4	45.37	33.94	20.80	39.80
5	43.23	28.09	51.23	27.80
6	28.09	36.94	31.40	23.94
7	38.51	33.67	41.44	46.89
8	43.66	34.29	33.44	38.67
9	37.66	28.33	33.78	38.44
10	21.56	32.44	40.78	38.44

Unit expressed as mg/ml

Table B10 Liver cytosolic protein concentration of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	82.97	30.97	50.72	68.05
2	82.22	92.69	56.30	80.88
3	71.72	96.05	105.72	76.63
4	92.72	95.49	67.30	65.54
5	73.97	80.63	71.20	63.91
6	79.30	106.34	68.91	64.77
7	48.38	79.06	41.63	78.63
8	85.34	95.97	78.26	78.83
9	77.34	63.26	81.69	59.49
10	57.06	100.26	97.40	80.34

Unit expressed as mg/ml

Table B11 Liver cytosolic protein concentration of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	49.80	58.30	42.72	34.63
2	36.72	29.80	65.63	51.22
3	62.05	50.72	41.13	61.63
4	23.83	24.69	35.54	46.11
5	42.91	38.49	38.55	56.26
6	50.49	39.77	54.38	42.55
7	48.91	52.91	43.91	60.06
8	49.77	41.54	35.77	39.49
9	37.40	44.11	44.26	60.26
10	58.54	32.97	43.26	53.20

Unit expressed as mg/ml

Table B12 Liver microsomal UDPGT activity of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	6.23	3.85	4.18	3.93
2	5.35	5.60	7.19	7.38
3	3.53	3.10	4.50	5.45
4	6.20	6.31	6.04	4.13
5	3.72	5.40	4.35	5.22
6	4.60	7.38	3.85	4.60
7	3.67	5.31	4.08	7.86
8	4.40	10.53	6.45	7.00
9	6.43	5.20	7.94	6.44
10	5.20	5.27	6.21	6.58
Mean	4.93	5.80	5.48	5.86
SEM	0.35	0.64	0.46	0.44

Unit expressed as nmol/min/mg protein

Table B13 Liver microsomal UDPGT activity of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	4.14	4.15	2.44	3.75
2	5.15	2.65	2.20	3.02
3	4.22	3.94	2.94	4.23
4	3.31	3.48	4.13	3.30
5	3.25	3.33	4.08	3.73
6	2.35	2.34	3.09	4.03
7	2.93	2.57	4.34	3.66
8	3.53	4.21	4.10	5.42
9	2.75	3.82	3.10	3.25
10	3.53	3.81	4.04	5.10
Mean	3.52	3.43	3.45	3.95
SEM	0.26	0.22	0.25	0.25

Unit expressed as nmol/min/mg protein

Table B14 Liver cytosolic SULT activity of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	6.98	8.97	6.99	5.72
2	7.11	5.80	8.32	2.37
3	10.09	6.18	7.25	3.35
4	10.98	3.89	7.54	1.39
5	10.72	3.35	2.23	2.98
6	9.24	4.30	2.88	5.38
7	10.55	5.50	7.56	3.84
8	2.75	1.78	2.33	3.15
9	2.24	2.15	1.67	5.09
10	10.16	1.93	1.87	1.91
Mean	8.08	4.39	4.86	3.52
SEM	1.03	0.72	0.90	0.47

Unit expressed as nmol/min/mg protein

Table B15 Liver cytosolic SULT activity of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	2.99	1.80	0.80	2.70
2	0.88	2.14	6.68	5.14
3	0.99	0.96	4.62	6.64
4	0.94	1.45	1.04	4.67
5	2.89	1.28	6.02	5.00
6	4.88	4.37	7.34	2.49
7	5.92	6.49	4.02	3.81
8	3.43	2.14	4.40	4.97
9	6.12	5.45	1.01	2.01
10	0.75	1.67	1.72	5.19
Mean	2.98	2.77	3.77	4.26
SEM	0.66	0.61	0.79	0.46

Unit expressed as nmol/min/mg protein

Table B16 Liver cytosolic GST activity of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	348.39	766.87	462.10	332.94
2	353.47	247.80	324.71	249.21
3	331.15	191.96	267.51	354.79
4	286.48	266.72	383.08	298.01
5	259.82	308.12	370.87	371.62
6	315.26	332.07	399.07	378.74
7	390.79	381.43	405.36	288.14
8	336.89	315.85	345.40	380.57
9	335.37	343.33	367.24	485.90
10	331.34	254.03	274.32	334.52
Mean	328.90	340.82	359.97	347.44
SEM	11.40	50.42	18.88	20.51

Unit expressed as nmol/min/mg protein

Table B17 Liver cytosolic GST activity of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	266.69	238.53	336.49	415.10
2	395.73	429.95	297.60	323.36
3	186.34	292.66	292.52	281.42
4	373.54	367.05	329.73	271.09
5	324.08	336.94	283.72	241.62
6	315.66	479.32	339.05	363.54
7	303.49	286.45	320.26	293.98
8	524.29	398.71	362.56	439.19
9	346.76	350.69	289.48	329.30
10	290.93	464.44	317.85	337.76
Mean	332.75	364.47	316.93	329.64
SEM	28.14	*25.10*	8.13	19.81

Unit expressed as nmol/min/mg protein

Table B18 Liver cytosolic NQOR activity of male rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	201.43	334.29	205.71	205.71
2	240.00	72.86	132.86	227.14
3	145.71	162.86	175.71	115.71
4	171.43	158.57	308.57	107.14
5	145.71	90.00	205.71	98.57
6	261.43	171.43	150.00	175.71
7	128.57	162.86	158.57	128.57
8	124.29	128.57	162.86	167.14
9	270.00	34.29	227.14	231.43
10	222.86	184.29	347.14	240.00
Mean	191.14	150.00	207.43	169.71
SEM	17.50	25.69	22.21	17.31

Unit expressed as nmol/min/mg protein

Table B19 Liver cytosolic NQOR activity of female rats

Rat No.	Control	Treatment groups		
		ECa 233 10 mg/kg/day	ECa 233 100 mg/kg/day	ECa 233 1,000 mg/kg/day
1	261.43	154.29	510.00	274.29
2	398.57	420.00	321.43	385.71
3	162.86	304.29	171.43	411.43
4	321.43	210.00	351.43	304.29
5	321.43	128.57	428.57	304.29
6	252.86	145.71	171.43	295.71
7	308.57	227.14	274.29	270.00
8	578.57	372.86	261.43	184.29
9	291.43	274.29	342.86	252.86
10	201.43	355.71	291.43	231.43
Mean	309.86	259.29	312.43	291.43
SEM	36.46	32.45	33.22	21.33

Unit expressed as nmol/min/mg protein

Appendix C

Vivid CYP450 Screening Kits Protocol

invitrogen™

Vivid® CYP450 Screening Kits Protocol

Cat. no. P2856, P2857, P2858, P2859, P2860, P2861, P2862, P2863, P2864, P2968, P2969, P2970, P2971, P2972, P3019, P3020 and P3021

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1.0 INTRODUCTION

Vivid® CYP450 Screening Kits enable rapid measurement of interactions between drug candidates and cytochrome P450 enzymes using a simple "mix-and-read" fluorescent assay that is designed for high-throughput screening in multiwell plates. These kits will allow investigators to rapidly identify compound-CYP450 interactions, eliminating unsuitable compounds early in the drug discovery process. Vivid® CYP450 Screening Kits can also be used to generate predictive structure-activity relationship models to guide medicinal chemists in their design of compounds.

Test compounds are analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP450 isozymes and specific Vivid® CYP450 Substrates. The availability of more than one structurally unrelated fluorogenic Vivid® CYP450 Substrate for CYP3A4, CYP3A5, CYP2C9, CYP2B6 and CYP2D6 reduces the potential for false negatives (and false positives) that could result from substrate-dependent interactions.

2.0 MATERIALS SUPPLIED

Vivid® CYP450 Screening Kit	Description	Cat. no.	Quantity	Storage
Vivid® CYP1A2 Blue (P2863)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP1A2 BACULOSOMES® Reagent	P2792	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Blue (P3019)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80 °C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Cyan (P3020)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Blue (P2861)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Green (P2860)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMF Substrate	P2869	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Red (P2859)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® OOMR Substrate	P2868	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP2C19 Blue (P2864)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C19 BACULOSOMES® Reagent	P2570	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Blue (P2972)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Cyan (P2862)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® MOBFC Substrate	P2871	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2E1 Blue (P3021)	Vivid® CYP450 Reaction Buffer III	P2949	50 ml	RT
	CYP2E1 BACULOSOMES® Reagent	P2948	1.0 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Blue (P2858)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Cyan (P2968)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Green (P2857)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Red (P2856)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMR Substrate	P2865	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Blue (P2970)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Cyan (P2971)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Green (P2969)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected

All kits also contain 0.5 ml Regeneration System, 100X (P2878, 333 mM Glucose-6-phosphate and 30 U/ml Glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate pH 8.0) and 0.5 ml NADP+ (P2879, 10 mM NADP+ in 100 mM potassium phosphate pH 8.0). Store both components at -80°C.

- The Vivid® CYP450 Reaction Buffers are 200 mM (Reaction buffer I), 100 mM (Reaction buffer II), or 400 mM (Reaction buffer III) potassium phosphate pH 8.0.
- CYP450 BACULOSOMES® Reagents consist of recombinant human Cytochrome P450 (1 µM) and rabbit NADPH P450 Reductase.
- The Vivid® Substrates and Standards are supplied as a dried film. Reconstitution is necessary before use.

2.1 Materials Required but not Supplied

- Multiwell black plates suitable for fluorescence measurements (Note: black-walled, clear bottom plates are needed for bottom-read fluorescent microplate readers). Invitrogen recommends using Costar #3915 non-treated plates
- Fluorescence plate reader with filters as described in Table 6
- Pipeting devices
- Reagent reservoir(s)
- Acetonitrile, anhydrous
- DMSO, reagent grade
- Deionized water
- Stop Reagent (CYP450 isozyme specific inhibitor) if performing an endpoint assay or in kinetic mode for the positive control of inhibition. For more information on inhibitors, see Section 7.0.

3.0 STORAGE AND STABILITY

Vivid® CYP450 Substrates and Fluorescent Standards are stable for at least six months when stored desiccated and protected from light at –20°C. For short-term storage, acetonitrile- or DMSO-based stock solutions should be stored in a desiccator at 4°C. Long-term storage requires that organic solutions be kept desiccated at –20°C. DMSO solutions are hygroscopic, and cold vials should be warmed to ambient temperature before opening. After opening, they should be capped promptly to avoid reagent dilution by absorbed moisture. The CYP450 BACULOSOMES® Reagent should be stored at -80°C. No significant decrease in activity (see enclosed Certificate of Analysis) was observed after 5 freeze/thaw cycles except for CYP2D6 which showed a 5% decrease. The Regeneration System should be stored at -80°C. Upon first thaw, aliquot into single use vials as the reagent should not be subjected to additional freeze/thaw cycles. The NADP+ should be stored at -80°C and is stable for at least 10 freeze/thaw cycles. Store protected from light. The Vivid® CYP450 Reaction Buffer (2X) can be stored at 4°C or room temperature.

4.0 ASSAY THEORY

Vivid® CYP450 Screening Kits are designed to assess metabolism and inhibition of the predominant human P450 isozymes involved in hepatic drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. The kits employ Vivid® CYP450 Substrates and CYP450 BACULOSOMES® Reagents. The CYP450 BACULOSOMES® Reagents are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase (CYP2E1 also contains human cytochrome b_5). CYP450 BACULOSOMES® Reagents offer a distinct advantage over human liver microsomes in that only one CYP450 enzyme is expressed, thereby preventing metabolism by other CYP450s. The Vivid® Substrates are metabolized by a specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions. Figure 1 schematically depicts the metabolism of a Vivid® CYP450 Substrate into a fluorescent metabolite. Note that the Vivid® Substrates have two potential sites for metabolism (indicated by arrows in Figure 1) and that oxidation at either site releases the highly fluorescent metabolite.

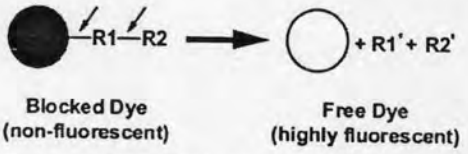

Figure 1. Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

The fluorescent metabolites are excited in the visible light spectrum, which minimizes interference caused by the background fluorescence of UV-excitable compounds and NADPH. The excellent reaction kinetics and optical properties of the Vivid® Substrates allow their use at concentrations at or below their K_m value in a reaction with P450 isozymes, assuring detection of even weak CYP450 inhibitors and providing the convenience of room temperature or 37°C incubations. The Vivid® CYP450 Assay may be run in a kinetic or endpoint mode (which is illustrated in Figure 2).

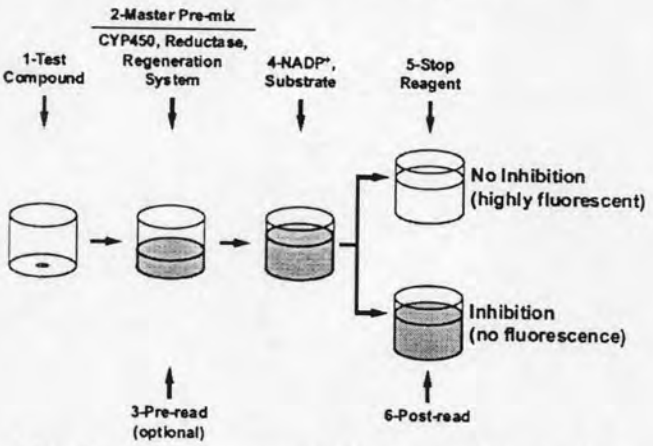

Figure 2. A schematic representation of an endpoint Vivid® CYP450 Assay

In end point (Section 5.1.9.2) mode, the test compounds (Step 1) are first combined with the Master Pre-mix (Step 2), consisting of CYP450 BACULOSOMES® Reagents and the Regeneration System (consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The Regeneration System converts $NADP^+$ into NADPH, which is required to start the CYP450 reaction. After a brief pre-incubation, the background fluorescence of the test compound and Master Pre-mix is measured (Step 3, pre-read). The enzymatic reaction is initiated by the addition of a mix of $NADP^+$ and the appropriate Vivid® Substrate (Step 4) and plate is incubated for the desired reaction time. After the addition of a Stop Reagent (Step 5), the fluorescence is measured in Step 6.

In kinetic mode (Section 5.1.9.1), the fluorescence is measured continuously starting after Step 4 (and eliminating Steps 5 and 6). Standard curves, constructed from the supplied Fluorescent Standard, can be used to calculate reaction rates from the observed fluorescence intensities in both assay formats. Assay parameters for isozymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 are listed in Tables 4 and 5.

5.0 VIVID® CYP450 HIGH-THROUGHPUT SCREENING ASSAY PROTOCOL

Each complete reaction must contain CYP450 BACULOSOMES® Reagent, Vivid® CYP450 Substrate, NADP+ and Regeneration System, all in the appropriate Vivid® CYP450 Reaction Buffer (supplied with each kit as a 2X solution). There are two possible modes for this assay: kinetic and endpoint. The method you choose will depend on your analytical needs and the equipment available. The kinetic mode is useful for analysis of one multiwell plate at a time and does not require the addition of the stop reagent. In endpoint mode, after an appropriate incubation time, the reaction is stopped by the addition of the CYP450 isozyme-specific inhibitor. Running in endpoint mode allows the reaction to be performed in several multiwell plates simultaneously.

Note: The following protocol is configured for use with one 96-well plate and 100 µl reactions. However, the protocol can be modified to accommodate several different plate formats by adjusting the calculations for the number of wells (and volume per well) in your experiment. See Trubetsoy *et al.* (2005) (see Section 9.0 for a complete list of references) for use of Vivid® kits in 1536-well plate formats. Each kit supplies enough reagents for at least 300 x 100 µl reactions.

5.1 Assay Procedure

5.1.1 Thaw Reagents

1. Thaw the P450 BACULOSOMES®, Regeneration System, and NADP+ on ice until ready to use. Do not vortex P450 BACULOSOMES® or Regeneration System.
2. Suggested assay conditions for screening with Vivid® kits are described in Table 1.

Table 1. Assay Conditions

Condition	Purpose	Dispensing
Test Compound	Screen for inhibition by compound of interest	40 µl 2.5X test compound 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Positive Inhibition Control	Inhibit the reaction with a known P450 inhibitor	40 µl 2.5X positive inhibition control (see Section 7.0) 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Solvent Control (No inhibitor)	Accounts for possible solvent inhibition caused by introduction of test compounds originally dissolved in an organic solvent such as DMSO	40 µl 2.5X solvent control 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Background	Enables subtraction of background fluorescence during data analysis	40 µl 2.5X solvent control 50 µl Vivid® CYP450 Reaction Buffer 10 µl Vivid Substrate and NADP+

5.1.2 *Reconstitution of Vivid® Substrate and Fluorescent Standard*

1. Reconstitute the Vivid® Standard using anhydrous acetonitrile and Fluorescent Standard using DMSO (see Tables 2 and 3).
2. Keep these solutions at room temperature for immediate use, or store at -20°C.

Table 2. Reconstitution of the Vivid® CYP450 substrates

Isozyme Type	Vivid® CYP450 Substrate	Molecular weight	mg per tube	µmol per tube	µl acetonitrile added per tube	[stock solution] (mM)	[screening concentration] (µM)
1A2	Vivid® EOMCC	245.2	0.1	0.41	205	2	3
2B6	Vivid® BOMCC	307.3	0.1	0.32	160	2	5
	Vivid® BOMFC	350.3	0.1	0.28	140	2	2
2C9	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMF	452.5	0.1	0.22	110	2	2
	Vivid® OOMR[1]	355.4	0.1	0.28	140	2	2
2C19	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
2D6	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
	Vivid® MOBFC	350.3	0.1	0.28	140	2	5
2E1	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
3A4	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2
	Vivid® BOMR	333.3	0.1	0.30	150	2	3
3A5	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2

[1] Heat at 70°C for 3-5 minutes and vortex to reconstitute.

Table 3. Reconstitution of the Fluorescent Standard. Use the blank cells in the table for your calculations. The value [X] is the amount of Assay Standard listed on the tube label.

Assay Standard	µmol per tube [X]	Reconstitution Solvent	µl Reconstitution Solvent added per tube [X x 10000]	[Fluorescent Standard] after Reconstitution, µM
Example	0.11	DMSO	1100 µl	100
Red Standard		DMSO/water (1:1)		100
Green Standard		DMSO		100
Blue Standard		DMSO		100
Cyan Standard		DMSO		100

5.1.3 *Prepare Standard Curve (Optional)*

1. With room temperature water, dilute enough Reaction Buffer (2X) to prepare enough 1X Reaction Buffer for your standard curve. In a 96-well plate, one standard curve can be run in 8 wells using 1 ml of Reaction Buffer. We recommend that at least six points (in addition to the blank) be used for the standard curve and that it be performed in duplicate.
2. To the first well of the column add 195 µl 1X Reaction Buffer.
3. Add 100 µl of 1X Reaction Buffer to each of the remaining wells in the column.
4. Add 5 µl of Fluorescent Standard (Table 3) to the first well containing 195 µl of buffer to achieve a starting concentration of 2.5 µM. Mix well.
5. Transfer 100 µl from this well into the next well containing 100 µl 1X Reaction Buffer and mix by pipetting. This is a two-fold dilution.
6. Repeat this dilution step, leaving the last well as an assay blank containing 1X Reaction Buffer only and no standard. The resulting Fluorescent Standard concentrations are: 2.5 µM, 1.25 µM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, 39.063 nM and 0 nM.

Note: These are suggested initial concentrations for the standard curve. More or less may be appropriate depending on you experimental needs.

Note: The assay can be performed simply using fluorescence values instead of converting to concentration of product formed.

5.1.4 *Prepare Test Compounds, Positive Inhibition Control, and Solvent Control*

1. Prepare 2.5X Test Compounds by dilution into deionized water. (For IC_{50} determination, a serial dilution of the test compound is required.)
2. Prepare a 2.5X solution of a known P450 Inhibitor in deionized water for positive control of inhibition (optional).

   Note: We recommend use of the inhibitors listed in Section 7.0.
3. Prepare a solution of the solvent used to dissolve the test compounds and known P450 inhibitor at 2.5X final concentration.

   Note: See Section 8.0 for information about particular solvents and tolerances.

5.1.5 *Dispense Test Compounds, Positive Inhibition Control, and Solvent Control*

1. Add 40 µl of the 2.5X solutions prepared in Section 5.1.4 to desired wells of the plate.
2. We recommend at least three replicates for the Positive Inhibition Control and Solvent Control.

5.1.6 *Prepare and Dispense Master Pre-Mix*

1. Prepare the Master Pre-Mix by diluting P450 BACULOSOMES® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer (2X) on ice (see Table 4). Mix by inversion.
2. Dispense 50 µl of Master Pre-Mix to each well. Mix.

   Note: To account for background fluorescence in the absence of CYP450 activity, dispense 50 µl of Vivid® CYP450 Reaction Buffer without P450 BACULOSOMES® to desired wells of the plate.

Table 4. Master Pre-mix (pre-mix of CYP450 BACULOSOMES® Reagents and Regeneration System). Keep on ice until ready to use

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Regeneration System (100X) added	µl of CYP450 BACULOSOMES® added	Concentration of CYP450 in Master Pre-mix (2X), nM	Screening concentration of CYP450, nM[1]
1A2	Vivid® EOMCC	4850 (Buffer I)	100	50	10	5
2B6	Vivid® BOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® BOMFC	4880 (Buffer I)	100	20	4	2
2C9	Vivid® BOMCC	4800 (Buffer II)	100	100	20	10
	Vivid® BOMF	4800 (Buffer II)	100	100	20	10
	Vivid® OOMR	4800 (Buffer II)	100	100	20	10
2C19	Vivid® EOMCC	4850 (Buffer II)	100	50	10	5
2D6	Vivid® EOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® MOBFC	4700 (Buffer I)	100	200	40	20
2E1	Vivid® EOMCC	4850 (Buffer III)	100	50	10	5
3A4	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5
	Vivid® BOMR	4850 (Buffer I)	100	50	10	5
3A5	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5

[1] For your first experiment, we suggest these concentrations of the CYP450 enzyme. Based on your results, you may find more or less enzyme is necessary.

5.1.7 *Pre-Incubate*

1 Incubate the plate for 20 minutes at room temperature to allow the compounds to interact with the CYP450 in the absence of enzyme turnover.

2. During this pre-incubation, prepare the pre-mixture of Vivid® Substrate and NADP+ (see Table 5).

3. You may also wish to include a pre-read at this point to determine if your compounds are fluorescent.

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Reconstituted Substrate added (Section 5.1.2)	µl of NADP+ (100X) added	Final % ACN from substrate
1A2	Vivid® EOMCC	885 (Buffer I)	15	100	0.15
2B6	Vivid® BOMCC	875 (Buffer I)	25	100	0.25
	Vivid® BOMFC	960 (Buffer I)	10	30	0.10
2C9	Vivid® BOMCC	850 (Buffer II)	50	100	0.50
	Vivid® BOMF	890 (Buffer II)	10	100	0.10
	Vivid® OOMR	890 (Buffer II)	10	100	0.10
2C19	Vivid® EOMCC	850 (Buffer II)	50	100	0.50
2D6	Vivid® EOMCC	850 (Buffer I)	50	100	0.50
	Vivid® MOBFC	945 (Buffer I)	25	30	0.25
2E1	Vivid® EOMCC	850 (Buffer III)	50	100	0.50
3A4	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10
	Vivid® BOMR	885 (Buffer I)	15	100	0.15
3A5	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10

5.1.8 *Start Reaction*

1. Start the reaction by adding 10 µl per well of the Vivid® Substrate and NADP+ mixture prepared in Step 5.1.7 and mix.

5.1.9 *Measure Fluorescence*

1. **Kinetic Assay Mode (recommended):** Immediately (less than 2 minutes) transfer the plate into the fluorescent plate reader and monitor fluorescence over time at excitation and emission wavelengths listed in Table 6.

2. **Endpoint Assay Mode:** Incubate the plate for the desired amount of time, then add 10 µl of recommended stop reagent (see Section 7.0) to each well to quench the reaction. Measure fluorescence in the fluorescent plate reader at excitation and emission wavelengths listed in Table 6.

   Note: Appropriate reaction times will vary by kit and experimental conditions. We recommend that you determine the linear activity range for the assay under the conditions you wish to use. Typically, such reaction times will fall within 5 to 60 minutes.

3. Proceed to Section 6.0 for data analysis.

Table 6. Recommended excitation and emission wavelengths and filter sets

		Vivid® Fluorescent Standard							
		Red		Blue		Green		Cyan	
Fluorescence Plate Readers	Excitation/ Emission	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width
with monochromators	excitation	530	--	409	--	485	--	400	--
	emission	585	--	460	--	530	--	502	--
using filters	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
with dichroic mirror	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
	dichroic	555	--	425	--	505	--	435	--

Red Standard is sodium salt of resorufin. Blue Standard is 3-cyano-7-hydroxycoumarin. Cyan Standard is 7-hydroxy-4-trifluoromethylcoumarin. We recommend exciting this dye off-peak at 400 nm (its excitation maximum is 385 nm) to minimize background from NADPH fluorescence. Green Standard is fluorescein.

For optimal signal to noise, filters must be blocked to OD of 6 outside their transparency range (UV and red blockage) and be free of pinholes. Filters may be purchased from:

Chroma Technology Corp.
72 Cotton Mill Hill, Unit A-9
Brattleboro, VT 05301
Phone: (800) 824-7662 or (802) 257-1800
Fax: (802) 257-9400.
www.chroma.com

6.0 SUGGESTED PROTOCOL FOR THE ANALYSIS OF RESULTS

6.1 Kinetic Assay Mode

1. Obtain reaction rates by calculating the change in fluorescence per unit time.
2. Calculate the percent inhibition due to presence of test compound or positive inhibition control using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{rate in presence of test compound or positive inhibition control}}{\text{rate in absence of test compound or positive inhibition control}} \right) \times 100\%$$

6.2 Endpoint Assay Mode

1. Subtract background fluorescence.
2. Calculate percent inhibition due to presence of test compound or positive inhibition using the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{RFU in presence of test compound or positive inhibition control}}{\text{RFU in absence of test compound or positive inhibition control}} \right) \times 100\%$$

Optional: Both types of data analysis above can be performed using a standard curve as described in Section 5.1.3 in order to calculate reaction rates as nmol product formed per unit time.

7.0 SUGGESTED CYP450 INHIBITORS (STOP REAGENT)

Table 7. Suggested CYP450 Inhibitors

Enzyme	Inhibitor (Stop Reagent)	Sigma-Aldrich Cat. no.	Suggested Final Concentration**
CYP1A2	α-naphthoflavone	N5757	3 µM
CYP2B6	miconazole	M3512	30 µM
CYP2C9	sulfaphenazole	S0758	10 µM
CYP2C19	miconazole	M3512	30 µM
CYP2D6	quinidine	Q3625	1 µM
CYP2E1	diethyldithiocarbamate	228680	100 µM
CYP3A4	ketoconazole	K1003	10 µM
CYP3A5	ketoconazole	K1003	30 µM

** To stop the reaction, the suggested final inhibitor concentration in the assay to produce inhibition of 90% or better is indicated in the above table. For an endpoint assay the volume of the added Stop Reagent should not exceed 10% of the final reaction volume [*e.g.*, 10 µl will be added per 100 µl reaction volume. This 10% increase in the volume of an endpoint reaction does not have a significant effect on the reaction (or the calculations)].

8.0 SOLVENT TOLERANCES

P450 activity can be inhibited by solvents commonly used to dissolve test compounds. While we always recommend including a solvent control in your experimental design, the following sample data is intended as a guide for the selection and use of organic solvents. Table values are percent inhibition at the indicated solvent concentration. Values preceded by a "+" indicate an increase in activity. Dashed lines indicate inhibition not detected. Note that lower concentrations are listed for 2E1 Blue; this isozyme is particularly sensitive to the presence of organic solvents.

Table 8. Solvent Tolerances

Vivid® Kit	Solvent concentration (%)	DMSO (% Inhibition)	Acetonitrile (% Inhibition)	Methanol (% Inhibition)	Ethanol (% Inhibition)
1A2 Blue	1	7	–	–	–
	0.1	–	–	–	–
	0.01	–	–	–	–
2B6 Blue	1	16	7	20	32
	0.1	–	–	–	–
	0.01	–	–	–	–
2B6 Cyan	1	–	–	–	9
	0.1	–	–	–	–
	0.01	–	–	–	–
2C9 Blue	1	55	9	46	61
	0.1	25	–	7	11
	0.01	–	–	–	–
2C9 Green	1	–	–	30	38
	0.1	–	–	–	–
	0.01	–	–	–	–
2C9 Red	1	21	5	45	53
	0.1	8	–	9	9
	0.01	5	–	–	–
2C19 Blue	1	23	–	21	42
	0.1	–	–	–	5
	0.01	–	–	–	–
2D6 Blue	1	58	–	37	56
	0.1	16	–	–	10
	0.01	4	–	–	–
2D6 Cyan	1	21	6	38	40
	0.1	–	–	4	5
	0.01	–	–	–	–
2E1 Blue	0.1	85	36	26	98
	0.01	75	15	3	75
	0.001	20	7	8	25
3A4 Blue	1	68	–	12	10
	0.1	25	–	4	–
	0.01	6	–	–	–
3A4 Cyan	1	68	4	20	11
	0.1	29	–	6	–
	0.01	7	–	–	–
3A4 Green	1	47	–	–	–
	0.1	9	–	–	–
	0.01	–	–	–	–
3A4 Red	1	48	–	6	5
	0.1	13	–	–	–
	0.01	–	–	–	–
3A5 Blue	1	73	5	29	32
	0.1	34	–	14	8
	0.01	8	–	5	6
3A5 Cyan	1	71	6	21	23
	0.1	30	–	8	6
	0.01	–	–	5	6
3A5 Green	1	15	–	–	–
	0.1	–	–	–	–
	0.01	–	–	–	–

9.0 REFERENCES

Cohen, L.H, *et al.* (2003) *In vitro* drug interactions of cytochrome P450: an evaluation of fluorogenic to conventional substrates. *Drug Metab. Dispos.* 31:1005-15.

Marks, B.D., *et al.* (2002) A high throughput screening assay to screen for CYP2E1 metabolism and inhibition using a fluorogenic Vivid® P450 substrate. *Assay Drug Dev. Technol.* 1:73-81.

Marks, B.D., *et al.* (2003) High-throughput screening assays for CYP2B6 metabolism and inhibition using fluorogenic Vivid® substrates. *AAPS PharmSci.* 5:E18.

Marks, B.D, *et al.* (2004) High-throughput screening assays for the assessment of CYP2C9*1, CYP2C9*2, and CYP2C9*3 metabolism using fluorogenic Vivid® substrates. *J. Biomol. Screen.* 9:439-49.

Trubetskoy, O.V., *et al.* (2005) Highly miniaturized formats for in vitro drug metabolism assays using Vivid® fluorescent substrates and recombinant human cytochrome P450 enzymes. *J. Biomol. Screen.* 10:56-66.

For structures of the Vivid Substrates and poster presentations containing additional details and applications of Vivid CYP450 Screening Kits, please visit us online at: www.invitrogen.com/drugdiscovery.

10.0 PURCHASER NOTIFICATION

Limited Use Label License No. 162: Cytochrome P450 enzymes, assays and substrates

This product is the subject of one or more of US Patent 5,891,696, 6,143,492, and 6,420,130. The purchase of this product conveys to the buyer the non-transferrable right to use the purchased amount o the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using the product or its components for Commercial Purposes. The buyer may transfer information or materials made through use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patent claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, 92008. Tel: (760) 603-7200. Fax: (760) 602-6500.

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Note: This study did not used protocol number 5.1.3, 6.1 and 7.0.

Appendix D

Study Protocol Approval by Chulalongkorn University Animal Care and Use Committee, Bangkok, Thailand

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 08-33-009	**Approval No. 08-33-009**
Protocol Title Effects of the standard extract of *Centella asiatica* (Eca 233) on human cytochrome P450 enzymes and phase II drug metabolizing enzymes in rats	
Principal Investigator Somsong Lawanprasert, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval March 24, 2008	**Date of Expiration** March 24, 2009
Applicant Faculty/Institution **Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330**	
Signature of Chairperson Withaya Janthasoot	**Signature of Authorized Official**
Name and Title **WITHAYA JANTHASOOT** **Chairman**	**Name and Title** **RUNGPETCH SAKULBUMRUNGSIL, Ph.D.** **Associate Dean (Research and Academic Service)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

Appendix E

Publication

Thai Journal of Pharmacology

www.phartherst.org

Official Publication of
Pharmacological and Therapeutic Society of Thailand

Proceedings of 30th Pharmacological and Therapeutic Society of Thailand Meeting

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P12 Effects of the Standard Extract of *Centella asiatica* (ECa 233) on Phase II Drug Metabolizing Enzymes in Rat Livers

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Abstract

Introduction: *Centella asiatica* (Linn.) Urban is widely used as food, beverage and traditional medicine. The standard extract of *C. asiatica* (ECa 233) has been studied for an indication of memory enhancer.

Objective: We investigated the effects of ECa 233 on the activity of hepatic phase II drug metabolizing enzymes using rats liver cytosols.

Materials and methods: Male Wistar albino rats were treated with either ECa 233 (10, 100 or 1,000 mg/kg/day, p.o.) or vehicle control for 90 days. Liver cytosols were prepared and used for analysis the activities of sulfotransferase (SULT), glutathione *S*-transferase (GST) and NAD(P)H Quinoneoxidoreductase (NQOR).

Results: The result demonstrated that both doses of ECa233 caused a decrease of SULT activity, whereas the activities of GST and NQOR were not changed.

Discussion and Conclusion: The inhibitory effect of ECa 233 on SULT activity suggested the possibility of drug interaction on medicines that are metabolized by this enzyme. No effects of ECa 233 on GST and NQOR activities suggested no advantage effect of this extract regarding the detoxification of xenobiotics via these enzymes.

Key words: *Centella asiatica*, Phase II Drug Metabolizing Enzymes

Introduction

Centella asiatica (Linn.) Urban is a traditional plant of which stems and leaves are used for preparing beverage, consumed as food and taken as traditional medicine. The major constituents are triterpenoids, mainly asiatic acid, asiaticoside, madecassic acid and madecassoside (1).

The standard extract of *C. asiatica* (ECa 233) has been studied preclinically for an indication of memory enhancer by Tantisira M. et al. at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. During research and development process of this extract for this indication, a study regarding effects of this extract on hepatic drug metabolizing enzymes either phase I and phase II metabolism are required. Modulation of this extract on hepatic phase I and phase II drug metabolizing enzymes would provide information regarding drug interaction and the possibility of the extract to increase/decrease risks of xenobiotic-induced toxicity/mutagenesis/carcinogenesis. Effect of ECa 233 on phase I enzymes (CYPs) has been studying. Thus, the aim of this study was to investigate effect of ECa 233 on hepatic phase II enzymes such as sulfotransferase (SULT), glutathione S-transferase (GST) and NAD(P)H quinoneoxidoreductase (NQOR) using rat livers cytosol.

Materials and Methods

Materials

These following chemicals were purchased from Sigma Chemical Co. Ltd., USA: adenosine 3'-phosphate 5'-phosphosufate (PAPS), bovine serum albumin, cupric sulfate, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenol-indophenol (DCPIP), dicumarol,

ethylenediaminetetraacetic acid (EDTA), Folin & Ciocalteu's phenol reagent, glutathione reduced from (GSH), magnesium chloride, nicotinamide adenine dinucleotide reduced from (NADH). Ethanol and potassium dihydrogen phosphate were purchased form Merck, Germany. 2-Naphthol was purchased from Aldrich, USA.

Animal treatment

Male Wistar albino rats (8 weeks old and weighing 250-300 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand. Rats were housed at the Department of Medical Sciences, Ministry of Public Health, Thailand. They were maintained at 22-25 °C with 12-h light/dark cycle and allowed free access to standard diet and water throughout the study. The animals were allowed to acclimatize for seven days before the study. Rats (10 per group) were randomly assigned to the various treatment groups. Rats were orally gavaged with ECa 233 at doses of 10, 100 or 1,000 mg/kg/day or water for 90 days. At the end of the extract administration, rats were euthanized by diethyl ether inhalation. Livers were perfused with ice-cold saline and removed. Liver cytosols were prepared by a differential centrifugation method (2) with some modification and stored at -80 °C until enzymes assays. Protein concentrations of liver cytosols were determined by the method of Lowry et al (1951) (3).

Enzymes assay

Cytosolic SULT activity was determined using the spectrophotometric method described by Frame et al. (2000) (4) with some modification. 2-Naphthol was used as a selective substrate for the assay.

Cytosolic GST activity was determined spectrophotometrically at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as a selective substrate according to the procedure of Habig et al. (1980) (5) with some modification.

Cytosolic NQOR activity was determined by the method modifies from the method of Ernster (1990) (6) using 2, 6-dichlorophenol-indophenol (DCPIP) as a selective substrate.

Statistical analysis. Data were presented as means $\pm$ SEM. Statistical differences were determined by one-way analysis of variance, followed by Student-Newman-Keuls test. $P < 0.05$ was chosen as indicating significance.

Results

A significant decrease in the activity of SULT was observed in rats treated with ECa 233 at all doses used in this study (10, 100 or 1,000 mg/kg/day) as compared to the control group (Figure 1).

ECa 233 given at all dosage regimens used in this study did not affect the activities of NQOR (Figure 2) and GST (Figure 3).

Discussion and Conclusion

The inhibitory effect of ECa 233 on SULT activity suggested the possibility of drug interaction of this extract on medicines that are metabolizes by this enzyme. No effects of ECa 233 on GST and NQOR activities suggested no advantage effect of this extract regarding the detoxification of xenobiotics via these enzymes.

Acknowledgements

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References

1. Inamdar PK, Yeole RD, Ghogare AB and Souza NJD. Determination of biologically active constituents in *Centella asiatica*. Journal of chromatography A.1996;742:127-130.
2. Lake BG, et al. Preparation and characterization of hepatic microsolmal and cytosolic fractions for studies on xenobiotic metabolism. In Biochemical Toxicology: A Practical Approach (K. Snell and B. Mullock, Eds.) IRL Press, Washington, DC. 1987:217-254.
3. Lowry Oh, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. J.Bio. Chem. 1951;193:265-275.
4. Frame LT, Ozawa S, Nowell SA, Chou HC, DeLongchamp RR, Doerge DR, Lang NP and Kadlubar FF. A simple colorimetric assay for phenotyping the major human thermostable phenol sulfotransferase (SULT1A1) using platelet cytosols. Drug Meab Dispos. 2000;28:1063-1068.
5. Habig WH, Pabst MJ and Jakoby WB. Glutathione *S*-Transferases. J. Biochemical Chemistry. 1974;249:7130-7139
6. Ernster L. DT diaphorase. Methods in Enzymology. 1990; 186: 287-301.

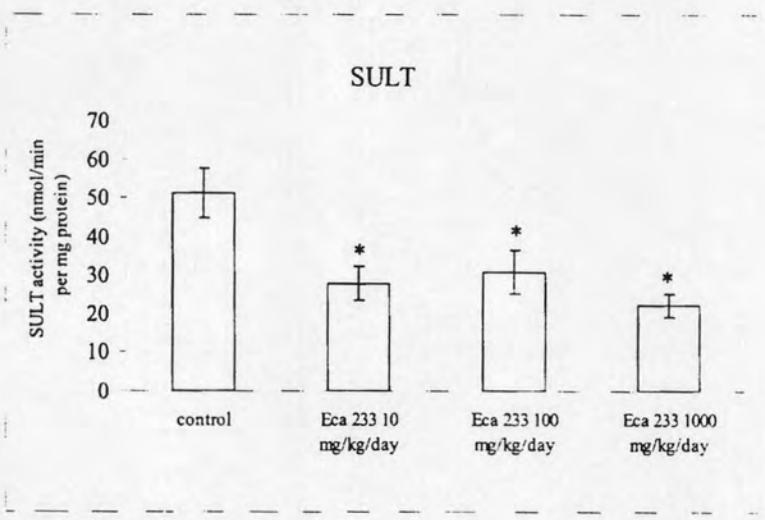

Figure 1 Effect of ECa 233 on hepatic cytosolic SULT activity. Data are presented as mean±SEM (n=10). * $P < 0.05$; ECa 233 treated group vs control group.

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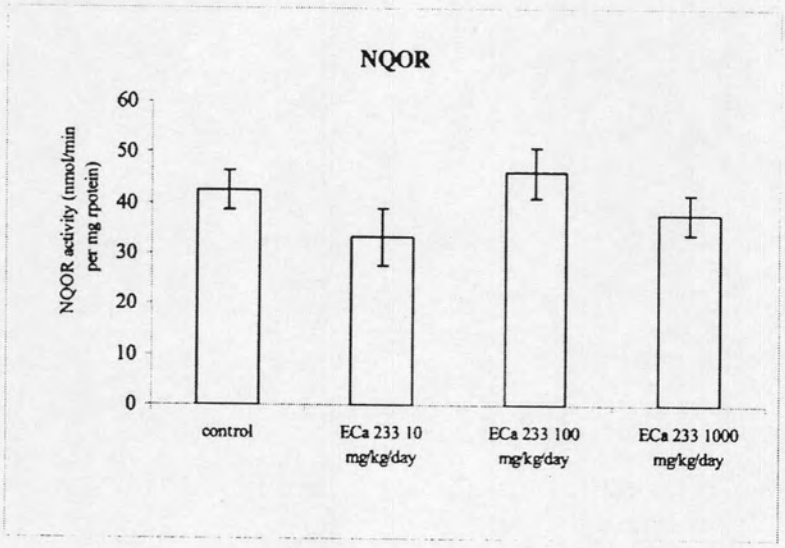

Figure 2 Effect of ECa 233 on hepatic cytosolic NQOR activity.

Data are presented as mean±SEM (n=10).

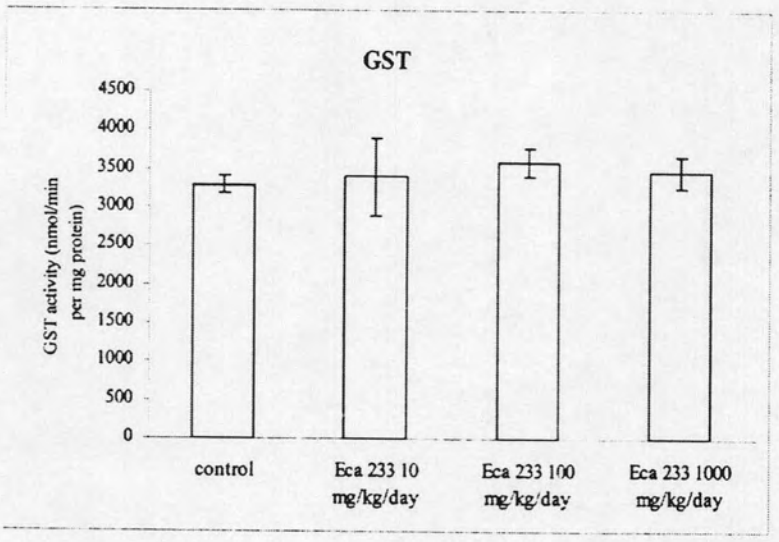

Figure 3 Effect of ECa 233 on hepatic cytosolic GST activity.
Data are presented as mean±SEM (n=10).

BIOGRAPHY

Miss Pitchayapa Seeka was born in March 14, 1975 in Yasothon, Thailand. She graduated with a Bachelor Degree of Pharmaceutical Sciences in 1999 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.