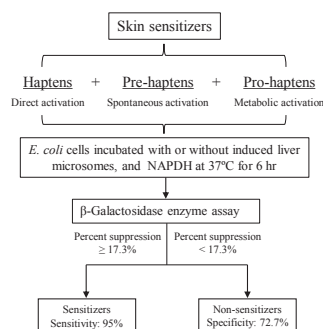


Identification of pre- and pro-haptens with a β -galactosidase-expressing *E. coli* culture system for skin sensitization

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ABSTRACT

Although several *in vitro* approaches were successful in separating chemicals as skin sensitizers and non-sensitizers, none of the available methods completely mimics the absolute *in vivo* scenario of skin sensitization. One of the major challenges with currently available systems would be the limited or no metabolic capacity to activate pre- or pro-haptens to reactive metabolites in the system. In the present study, *E. coli* cells with β -galactosidase-expressing *LacZ* gene were combined with either induced rat liver S-9 fractions or microsomal fractions to detect pre- or pro-haptens to cause skin sensitization. Following optimization of some experimental conditions, we examined 20 sensitizers classified as pre- or pro-haptens and 11 non-sensitizers in these *E. coli* cultures by incubating bacterial cells and test chemicals with and without S-9 or microsomal proteins. After a 6-h incubation in the presence of IPTG, cells were lysed to determine the suppression of β -galactosidase enzyme. A cut-off of 17.3% was applied to determine the percent suppression of β -galactosidase activity by test chemicals to classify skin sensitizers and non-sensitizers. Among chemicals tested, 19 pre- or pro-haptens were categorized as true positives and 8 non-sensitizers were categorized as true negatives. Thereby, the overall sensitivity, specificity and accuracy achieved with microsome-incorporated and S-9 fraction-incorporated group were 95.0%, 72.7% and 87.1% and 80.0%, 81.8% and 80.6%, respectively. The results suggested that the present bacterial system incorporated with the microsomal activation system could be considered as a useful alternative method to classify not only direct-acting sensitizers but also pre- or pro-haptens requiring metabolic activation *in vitro*.

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1. Introduction

With the discovery of operon model, the *LacZ* gene expressing β -galactosidase enzyme has always been an important aspect in molecular toxicology (Juers et al., 2012). Because of the large tetrameric structure of β -galactosidase enzyme and rapid induction by isopropyl β -D-1-thiogalactopyranoside (IPTG), toxic insults by certain chemicals may significantly affect its expression and enzymatic functions (Juers et al., 2012). As the interaction of reactive skin sensitizers with carrier proteins at the beginning of sensitization process, the interaction of β -galactosidase-expressing *E. coli* with reactive toxins might result in disturbance, slow or no production of β -galactosidase (Bitton and Koopman, 1992; Nepal et al., 2018a). Moreover, presence of nucleophilic pockets at Glu537 of β -galactosidase might support the possible interaction with reactive electrophiles (Yuan et al., 1994).

As an animal model, local lymph node assay (LLNA) has been considered as a gold standard to determine chemicals as skin sensitizers and non-sensitizers, where test chemicals were applied repeatedly on animal's ear to elicit chemical specific T-cell proliferation in the local lymph node (Kimber et al., 1991). After the OECD adopted 3Rs concept and European Union promulgated the rule of using non-animal models to test cosmetic ingredients, several *in vitro* models for skin sensitization, such as *in chemico*, hCLAT, and U-Sens were proposed as alternatives to *in vivo* (Ashikaga et al., 2006; Gerberick et al., 2007; Nepal et al., 2018b; Urbisch et al., 2015). When a test chemical is applied onto the skin, immature Langerhans cells (LC) present in epidermis would recognize the chemical and bind to the haptens to form LC-hapten complex (Gerberick et al., 2007). Then, LC-hapten complex would migrate from skin to draining lymph nodes where the chemical-specific T cells would proliferate and release into the circulation. After subsequent skin exposure to the same chemical, clinical manifestation of skin sensitization could be observed (Agüero et al., 2012; Nepal et al., 2018b). During the interaction of sensitizers with proteins, some haptens can directly bind to skin protein, whereas some need spontaneous air-oxidation or enzymatic metabolic activation to become reactive haptens, so called pre- or pro-haptens (Gerberick et al., 2009; Troutman et al., 2011). Although several *in vitro* approaches were successful in separating chemicals as skin sensitizers and non-sensitizers, none of the methods completely mimics the absolute *in vivo* scenario of skin sensitization, specifically to detect pre- or pro-haptens. Based on the previous reports, more than 25% of the total chemicals tested for skin sensitization would be pre- or pro-haptens, indicating that an alternative test incorporated with metabolic activation system would be required for improving predictability of the method (Patlewicz et al., 2016). Although an effort to incorporate peroxidase-peroxide oxidation system to convert chemicals to reactive haptens has been made, methods that can sufficiently categorize pre- or pro-haptens as sensitizers in simple and easy manners are still necessary (Gerberick et al., 2009).

Liver is the house for a variety of xenobiotic-metabolizing enzymes that produce reactive oxygen species as well as reactive metabolites (Johansson et al., 2009; Noh et al., 2017; Puntarulo and Cederbaum, 1998). When the enzymes act upon pre- or pro-haptens, the chemicals would be activated to reactive metabolites to cause skin sensitization (Johansson et al., 2009). In this regard, upon biotransformation of pre- or pro-haptens by hepatic enzymes, the reactive metabolites would act upon β -galactosidase expression system in *E. coli*, and then the production of the enzyme would be suppressed. Alternatively, they could also directly affect the activity of expressed β -galactosidase. By either way, the β -galactosidase activity reduced by test chemicals would be a toxic marker in the skin sensitization test, as we proposed in the previous report (Nepal et al., 2018a).

Although there are very limited literatures for CYP-mediated activation of topically applied chemicals (Natsch et al., 2013), it is highly evident that the metabolic activation might be necessary for identifying those pre- or pro-haptens from *in vitro* alternative tests where metabolic

activation capacity is very limited. Skin S-9 or microsomal fractions would be the best choice for the activation of sensitizers in the test. However, it is very complex to extract S-9 fractions or microsomes from skin for the test development, and the metabolic capacity of skin microsomes would not be high enough for activating sensitizers during the short period of chemical exposure time in any alternative tests available. In this regard, even in the well-known Ames test, Aroclor 1254-induced rat liver S-9 fractions are adopted for the maximum induction of xenobiotic-metabolizing enzymes including CYPs. Therefore, for a testing system that is readily performed for the identification of toxic signs following activation, more general metabolic activation system would be preferable than skin microsomes in the test. Because the purpose of proposed test here would be to better determine the sensitizing potential of certain chemicals that require metabolic activation, liver metabolic system was selected in the present study.

In our recent study, we developed a method to screen the skin sensitization potential of chemicals by measuring percent suppression of β -galactosidase activity by test chemicals in *E. coli* cells containing β -galactosidase-expressing *LacZ* gene in the presence of IPTG (Nepal et al., 2018a). It was thought that, by IPTG induction, the rapid expression of β -galactosidase in *E. coli* cells would be vulnerable to toxic insults by test chemicals, so that reactive sensitizers would suppress directly or indirectly on the expression of β -galactosidase as the sensitizer interacts with carrier proteins. From the total numbers of 33 chemicals with 22 sensitizers (weak to extreme) and 11 non-sensitizers, the accuracy of 87.7% was achieved (Nepal et al., 2018a). Nonetheless, two strong pre- and pro-haptens, hydroquinone and 2-aminophenol, were falsely classified as non-sensitizers in the study (Nepal et al., 2018a). In the present study, we investigated the applicability of *E. coli* cells with β -galactosidase-expressing system combined with either induced rat liver S-9 fractions or microsomal fractions to identify pre- and pro-haptens, based on their skin sensitizing and non-sensitizing potential from LLNA.

2. Materials and methods

2.1. Materials

Test chemicals with their suppliers are listed in Table 1. In addition, IPTG was obtained from A.G. Scientific (San Diego, CA, USA) and ortho-2-nitrophenyl β -D-galactopyranoside (ONPG) from Tokyo Chemical Industries (Tokyo, Japan). 3-Methylcholanthrene (3-MC), dexamethasone, acetone, chloroform, potassium chloride, and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate anhydrous, potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate monobasic, sodium phosphate dibasic, and magnesium sulfate were purchased from Duksan Pure Chemicals (Seoul, South Korea). Ampicillin-resistant *E. coli* serotype XL1-Blue carrying pUC18 vector with *LacZ* gene was obtained from Promega (Madison, WI, USA). The vector construct in detail is available in our previous report (Nepal et al., 2018a).

2.2. Animals

Male Sprague-Dawley (SD) rats with average weight of ~ 270 g were purchased from Orient Bio (Seoul, Korea) for the preparation of liver microsomes. The animals were housed two per cage and acclimatized for at least 1 week. The animal room temperature and relative humidity was maintained at $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$, respectively. The air change and light intensity was maintained at 10–20 cycles/h and 150–300 lux with a 12-h light/dark cycle. An approval for animal use was issued from Yeungnam University Animal Care and Use Committee (approved No., 2014-008).

Table 1
General information of test chemicals used in the present study.

No.	Chemicals	CAS No.	LLNA category	EC3 (%)	Chemical suppliers	Purity (%)	Physical state	Metabolic pathways or enzymes	Metabolites identified	References
1	p-Hydroquinone	123-31-9	Strong	0.11	Sigma	> 99	Solid	Peroxidase	1,4-Benzoquinone	Kettle and Winterbourn (1992)
2	2-Aminophenol	591-27-5	Strong	0.40	Sigma	99	Solid	Tyrosinase	2-Aminophenoxazine-3-one	Washington et al. (2015)
3	p-Phenylenediamine (PPD)	95-54-5	Strong	0.06	Sigma	> 99	Solid	N-Acetylation, N-hydroxylation	N-Acetyl-PPD and N,N'-diacetyl-PPD	Hu et al. (2009)
4	Benzo(a)pyrene	50-32-8	Strong	0.225	Sigma	98	Solid	CYP1A1	Epoxides	Shiizaki et al. (2017)
5	CD3	25646-77-9	Strong	0.60	TCI	> 98	Solid	N/A	N/A	N/A
6	Metol	55-55-0	Strong	0.61	TCI	> 98	Solid	N/A	N/A	N/A
7	Lauryl gallate	1166-52-5	Strong	0.30	TCI	N/A	Solid	CYP3A1	Gallic acid	Booth et al. (1959), Nakagawa et al. (1995)
8	Propyl gallate	121-79-9	Strong	0.32	TCI	> 98	Solid	CYP3A1	Gallic acid	Booth et al. (1959), Nakagawa et al. (1995)
9	Isoeugenol	97-54-1	Moderate	1.30	TCI	> 97	Liquid	N/A	N/A	N/A
10	3-Aminophenol	591-27-5	Moderate	3.20	Fluka	> 98	Solid	N/A	4-Aminocatechol, m-aminophenyl sulfate	Goodwin (1976)
11	1-Naphthol	90-15-3	Moderate	1.30	TCI	> 99	Solid	CYP1A2, CYP2D6*1	1,4-Naphthoquinone	Cho et al. (2006)
12	4-Amino-m-cresol	2835-99-6	Moderate	2.15	TCI	> 98	Solid	N/A	N/A	N/A
13	Methyl eugenol	93-15-2	Moderate	2.40	TCI	> 98	Liquid	CYP2E1, CYP1A2, CYP2B	2,3'-Hydroxymethylisoeugenol, 6-Hydroxymethyleugenol	Cartus et al. (2012)
14	Farnesol	4602-84-0	Moderate	4.10	TCI	N/A	Liquid	CYP1A, CYP2B, CYP3A / UGTs 1A1, 2B7	Farnesyl glucuronide, Hydroxyfarnesol, Hydroxyfarnesyl glucuronide	Špičáková et al. (2017), Staines et al. (2004)
15	Eugenol	97-53-0	Weak	13.00	TCI	> 99	Liquid	CYP1A, Phase 2 conjugation	Sulfate, glucuronic acid and glutathione conjugation	Han et al. (2007), Thompson et al. (1991)
16	Benzocaine	94-09-7	Weak	22.03	Sigma	N/A	Solid	CYP1A2, 2B6, 2C19, 2D6, 2E1	Methemoglobin	Hartman et al. (2014)
17	Cinnamic alcohol	104-54-1	Weak	21.00	TCI	> 97	Liquid	CYP2E1 or alcohol dehydrogenase	Cinnamaldehyde	Smith et al. (2000)
18	Aniline	62-53-3	Weak	89.00	Sigma	> 99.5	Liquid	CYP2E1	p-Aminophenol	Myers et al. (2001)
19	(R)-(+)-Limonene	5989-27-5	Weak	69.00	Sigma	97	Liquid	CYP2C9, 2C19	Carveols and perillyl alcohol	Miyazawa et al. (2002)
20	Geraniol	106-24-1	Weak	26.00	Sigma	98	Liquid	CYP1A1, CYP3A5, CYP2B6	Geranal	Hagvall et al. (2008)
21	Methyl salicylate	119-36-8	Non-sensitizer	NC	TCI	> 99	Liquid	Hydrolysis	Free salicylate	Davison et al. (1961)
22	1-Butanol	71-36-3	Non-sensitizer	NC	Fluka	N/A	Liquid	Alcohol dehydrogenase	Carboxylic acids	Plapp et al. (2015)
23	Salicylic acid	69-72-7	Non-sensitizer	NC	Sigma	99	Solid	CYP2E1, CYP3A4, hydroxylation and decarboxylation	2,3-Dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid to catechol	Bojić et al. (2015), Ohmoto et al. (1991), Stumpf (2016)
24	Lactic acid	50-21-5	Non-sensitizer	NC	Sigma	85	Liquid	N/A	N/A	N/A
25	Glycerol	56-81-5	Non-sensitizer	NC	Sigma	> 99	Liquid	N/A	N/A	N/A
26	2-Propanol	67-63-0	Non-sensitizer	NC	Sigma	> 99.5	Liquid	Alcohol dehydrogenase	Acetone	Plapp et al. (2015)
27	4-Hydroxybenzoic acid	99-96-7	Non-sensitizer	NC	Sigma	> 99	Solid	Hydroxylation and decarboxylation	3,4-Dihydroxybenzoic acid to hydroquinone	Stumpf (2016)
28	Tween 80	9005-65-6	Non-sensitizer	NC	Sigma-Aldrich	N/A	Liquid	N/A	N/A	N/A
29	Sulfanilamide	63-74-1	Non-sensitizer	NC	TCI	N/A	Solid	Acetylation	N-Acetyl and N-formyl derivatives	Morland and Olsen (1977)
30	Vanillin	121-33-5	Non-sensitizer	NC	Sigma-Aldrich	99	Solid	CYP2E1, CYP1A2	Vanillic acid, vanillyl alcohol, catechol	Chen et al. (2012), Strand and Scheline (1975)
31	4-Aminobenzoic acid	150-13-0	Non-sensitizer	NC	TCI	> 99	Solid	CYP3A4	p-Acetamidobenzoic acid	Davis et al. (2011), Hung et al. (1987)

NC, Not calculated; N/A, not available.

2.3. Preparation of induced rat liver S-9 fractions and microsomes

S-9 fractions and microsomes were prepared as described previously (Noh et al., 2015). In brief, acclimatized animals were pre-treated with either 3-methylcholanthrene (3-MC) at 40 mg/kg for 3 days, phenobarbital at 80 mg/kg for 3 days, dexamethasone at 50 mg/kg for 3 days, or acetone at 5 ml/kg for 2 days. Acetone was administered orally, whereas 3-MC, phenobarbital and dexamethasone were administered intraperitoneally. After 24-h of last treatment, rats were sacrificed and livers were collected. Livers were homogenized with four volumes of sterilized 0.1 M potassium phosphate buffer, pH 7.4, followed by a centrifugation at $9000 \times g$ at 4 °C for 20 min to prepare S-9 fractions. The aliquots were prepared and stored at –70 °C until their use. To prepare microsomal fractions, the S-9 fractions were collected into another tube and further centrifuged at $105,000 \times g$ at 4 °C for 1 h. The microsomal pellets were re-suspended in sterilized 0.1 M potassium phosphate buffer, pH 7.4. Individual S-9 fractions and microsomes prepared separately were pooled in equal volumes to prepare a metabolic activation system and aliquots were stored at –70 °C until use.

2.4. Determination of CYP activities

Monoxygenase activities, such as ethoxyresorufin O-deethylase (EROD), pentoxyresorufin O-depentylase (PROD), p-nitrophenol hydroxylase (PNPH), and erythromycin N-demethylase (ERDM) were determined with the same procedure as previously described (Noh et al., 2015). The enzyme activities of uninduced, individually induced, and pooled induced liver S-9 fractions or microsomes were compared. The protein contents of liver S-9 fractions and microsomes were determined by using bovine serum albumin as a standard (Lowry et al., 1951).

2.5. Incubation of bacterial cells with test chemicals

E. coli cells were grown in Luria-Bertani (LB) medium prepared by mixing casein enzyme hydrolysate (10 g/l), yeast extract (5 g/l) and sodium chloride (5 g/l) in distilled water and the pH of the medium was adjusted to 7.0. The media was autoclaved at 121 °C for 15 min and stored at 4 °C until use. The bacterial stock containing *LacZ* gene was separated by the blue-white colony screening technique as described previously (Nepal et al., 2018a). From a single bacterial colony, a stock was prepared and stored at 4 °C. To make the bacterial culture ready for the β -galactosidase suppression by test chemicals, 20 μ l of bacterial stock was mixed with 5 ml of broth and incubated at 37 °C to obtain the optimal OD₆₀₀ value of 0.5–0.6. Once OD₆₀₀ value reached 0.5–0.6, the bacterial culture was used for the incubation with a test chemical (Nepal et al., 2018a).

2.6. Preparation of reaction mixture

Chemical stocks were prepared in acetonitrile. DMSO was used to dissolve the chemicals if the chemicals were not dissolved in acetonitrile. In Eppendorf tubes, 2.0 μ l of either 100 mM, 150 mM, or 200 mM of chemical stock solutions was mixed with 408.0 μ l of LB broth containing 30 μ M of IPTG and 60 μ l of either pooled liver S-9 fractions or microsomes to make the final concentrations of chemicals to be 0.4 mM, 0.6 mM, and 0.8 mM, respectively. After the contents of tubes were vortex-mixed, 20 μ l of *E. coli* cells with OD₆₀₀ of 0.5–0.6 and 10 μ l of NADPH at 50 mM (1 mM final concentration) prepared in potassium phosphate buffer, pH 7.4, were added into each tube to be a total volume of 0.5 ml. The samples were mixed by a tube inversion technique and incubated at 37 °C for 6 h in a shaking incubator at 180 rpm. The group without either S-9 or microsome fractions was also prepared in parallel, in which the corresponding volume of potassium phosphate buffer, pH 7.4, was used instead of either S-9 fractions or microsomes and NADPH. Control samples were prepared for individual concentrations of chemicals that include IPTG containing broth, S-9 or

microsomes, *E. coli* cells and NADPH. Acetonitrile, 2 μ l, was added instead of a test chemical into the control sample tubes. Tubes containing all except IPTG was used as the blank for the experiment. To ensure no bacterial contamination in media and buffers, a negative control containing only broth and potassium phosphate buffer, pH 7.4, was prepared and incubated together with other samples. Subsequent to incubation, β -galactosidase activity was determined.

2.7. Measurement of β -galactosidase activity

β -Galactosidase activity was measured with the similar process as described in our previous study (Nepal et al., 2018a). Briefly, after the 6-h incubation of samples, 100 μ l of chloroform and 50 μ l of 0.1% SDS were added into the tubes and vigorously vortexed to lyse the cells. To determine the enzyme activity, 400 μ l of ONPG at 2 mg/ml prepared in Z-buffer, pH 7.5, was added into the tubes and vortexed. Samples were incubated at 37 °C for 30 min and the reaction was terminated by adding 200 μ l of 1 M Na₂CO₃. Finally, the samples were centrifuged and 200 μ l of supernatant was transferred to a 96-well plate and the intensity of the o-nitrophenol formed was measured at 420 nm by a UV-spectrophotometer (Tecan Spark 10 M, Austria). The light scattering by *E. coli* cells at 420 nm were corrected by measuring absorbance at 550 nm. The β -galactosidase activity in Miller unit was determined with the following formula: $1000 \times OD_{420} - (1.75 \times OD_{550}) / (T \times \text{cell volume} \times OD_{600})$, where OD₄₂₀ was the absorbance at 420 nm, OD₅₅₀ was the absorbance at 550 nm, T was the time required to develop the yellow color, and OD₆₀₀ was the absorbance value at 600 nm (Nepal et al., 2018a). Thereafter, the percent suppression of β -galactosidase activity was determined as indicated in our previous report (Nepal et al., 2018a).

3. Results

In our previous study, we investigated suppressive effects of 22 skin sensitizers and 11 non-sensitizers on the β -galactosidase activity (Nepal et al., 2018a). Chemicals were predicted as skin sensitizers or non-sensitizers based on percent suppression of β -galactosidase by test chemicals in which a cut-off percent of 17.3 was applied. As mentioned, two LLNA categorized sensitizers, p-hydroquinone and 2-aminophenol, which were known as pre- or pro-haptens, were determined to be false negatives. To accurately predict the sensitizing potential of certain chemicals, a system that could detect not only direct acting sensitizers but also pre- or pro-haptens must exist. Therefore, a system incorporated with either S-9 fractions- or microsome-mediated metabolic activation system was designed in the present study. The percent suppression of β -galactosidase by 31 chemicals were studied in the current study, where 20 chemicals of either pre- or pro-haptens were selected from literatures as listed in Table 1. Eleven non-sensitizers were the same as we studied previously for testing whether the results would be the same as in the direct incubation system when metabolic activation systems are supplemented (Nepal et al., 2018a).

Prior to proceeding to the main tests, essential conditions were optimized. At first, we tested whether the parameters that were successful in inducing *LacZ* gene for the production of β -galactosidase enzymes in *E. coli* culture system in our previous study would be sufficient to induce *LacZ* gene in the current microsome-incorporated system (Nepal et al., 2018a). To do so, parameters, such as concentration dependencies of IPTG and ONPG along with their pH requirements for the maximum induction of β -galactosidase enzymes were determined. Consistent with the previous study, IPTG at 30 μ M in LB media, pH 7.0, and ONPG at 2 mg/ml in Z-buffer, pH 7.5, were noticed to be sufficient for the induction of *LacZ* gene to produce β -galactosidase enzyme (Fig. 1). The results indicated that the incorporation of microsomal fraction in *E. coli* cultures would not significantly affect the results. The addition of S-9 fractions also gave no interferences (data not shown).

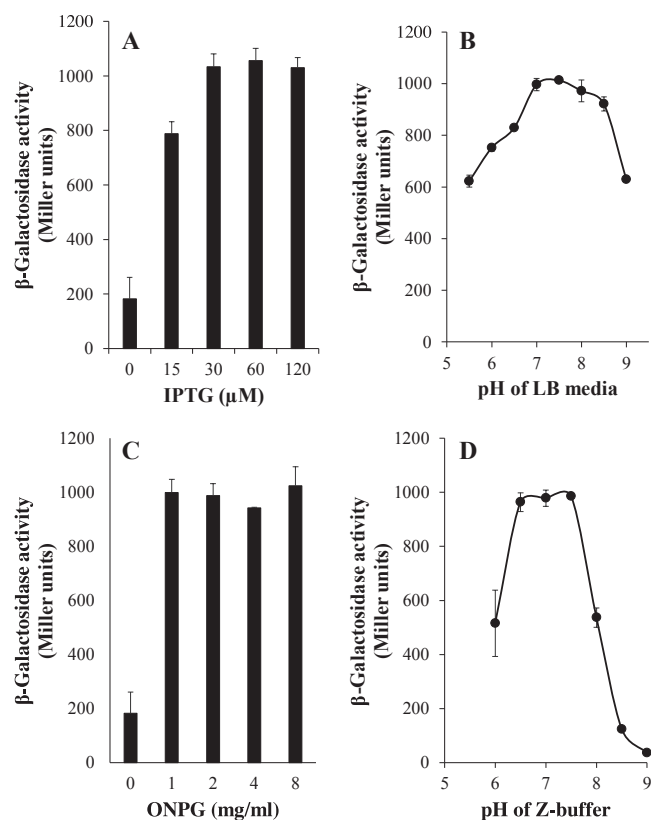


Fig. 1. Optimization of experimental conditions. (A) IPTG concentration dependency. *E. coli* cells at OD_{600} of 0.5–0.6 were added into the mixture of LB broth, pH 7.0, containing various concentrations of IPTG and 2 mg/ml of pooled liver microsomes induced by 4 inducers, in a total volume of 500 μ l. The contents were mixed and incubated at 37 $^{\circ}$ C for 6 h and β -galactosidase enzyme activity was determined with 2 mg/ml of ONPG prepared in Z-buffer, pH 7.5. Each bar represents the mean activity of β -galactosidase + SD of triplicate determinations. (B) Effects of LB medium pH on β -galactosidase activity. *E. coli* cells at OD_{600} of 0.5–0.6 were added into LB broths of various pHs containing 30 μ M IPTG and 2 mg/ml of pooled microsomes. After mixing, samples were incubated at 37 $^{\circ}$ C for 6 h and β -galactosidase enzyme activity was determined. Each point represents the mean activity of β -galactosidase $\pm$ SD of triplicate determinations. (C) ONPG concentration dependency. *E. coli* cells were added into LB broth, pH 7.0, containing 30 μ M IPTG and 2 mg/ml pooled microsomes. After mixing, samples were incubated at 37 $^{\circ}$ C for 6 h and β -galactosidase enzyme activity was determined with various concentrations of ONPG prepared in Z-buffer of pH 7.5. Each bar represents the mean activity of β -galactosidase + SD of triplicate determinations. (D) Effects of pH of Z-buffer on ONPG activity. *E. coli* cells were added into LB broth, pH 7.0, containing 30 μ M IPTG and 2 mg/ml pooled microsomes. After mixing, samples were incubated at 37 $^{\circ}$ C for 6 h and β -galactosidase enzyme activity was determined with 2 mg/ml of ONPG prepared in Z-buffer with various pHs. Each point represents the mean activity of β -galactosidase $\pm$ SD of triplicate determinations.

Thereafter, to investigate the applicability of test method incorporated with metabolic activation system in the previously developed system with *E. coli* cultures, several pre- and pro-haptens were preliminarily tested. At first, the effects of either uninduced S-9 fraction or microsome were compared with either induced S-9 fraction or microsome. The percent suppression of β -galactosidase by several pre- or pro-haptens following the activation with induced S-9 or microsomes were much higher than uninduced S-9 or microsomes, indicating some sensitizers would require metabolic activation (data not shown). Therefore, the induced fraction of either S-9 or microsomes were chosen for the subsequent experiments. Next, the percent suppression of β -galactosidase by pre- or pro-haptens in the presence of pooled liver S-9 fractions or microsomes induced by individual inducers was investigated. The induced fractions were originally prepared for the

xenobiotic metabolism studies (Noh et al., 2015). Because the individual inducer-induced microsomes were thought to have high activities of only few xenobiotic-metabolizing enzymes, we decided to use pooled liver fractions to use pan CYP-induced metabolic activation system.

In the test system, *E. coli* cells with optimal cell densities (OD_{600} 0.5–0.6) were added into LB broth medium, pH 7.0, containing 30 μ M of IPTG and treated with various test chemicals at 0.6 mM. Either induced liver microsomes at 2 mg/ml, S-9 fractions at 2.5 mg/ml or potassium phosphate buffer, pH 7.4, as control was added into the incubation mixture along with 1 mM of NADPH. After a 6-h incubation, the percent suppression of β -galactosidase was determined colorimetrically by using 2 mg/ml of ONPG. As shown in Fig. 2, the percent suppression of β -galactosidase by test chemicals, such as p-hydroquinone, 2-aminophenol, and 3-aminophenol, were much higher with both pooled microsome- and S-9 fraction-added groups than with control groups. Likewise, when we compared the percent suppression of β -galactosidase between microsome-added and S-9-added groups, except 2-aminophenol, a higher percent suppression was observed with pooled microsome-added group. 2-Aminophenol showed higher percent suppression with S-9 fractions than microsomes, which signified that the enzymes responsible for metabolic activation of 2-aminophenol would reside in S-9 fractions than in microsomal fractions. Conversely, no differences were observed with chemicals, such as isoeugenol and geraniol, regardless of with or without the addition of liver fractions, indicating that these pre- or pro-haptens would be strong enough to cause sensitization by themselves. Likewise, the addition of either S-9 fractions or microsomes in *E. coli* cultures did not affect the results on non-sensitizers, lactic acid and 2-propyl alcohol. The results also indicated that the incorporation of metabolic activation system in *E. coli* cultures could be applicable to improve predictive capacity for identifying skin sensitizers requiring metabolism. Because of the better results with microsomes, we primarily chose pooled induced microsome rather than S-9 fractions for the subsequent experiments. However, it would be apparent that several additional metabolic enzymes along with specific CYP enzymes would exist in S-9 fractions. Therefore, it was worth to explore the effects of S-9 fractions on chemicals that would only be responsive to certain enzymes besides CYP enzymes. Therefore, pooled induced S-9 fractions were also tested in the present study.

To ensure the induction of CYP enzymes by several inducers, specific monooxygenase activities of variously induced, uninduced and pooled liver microsomes were compared (Fig. 3). Although 3-MC, phenobarbital, acetone, and dexamethasone could induce CYP1A-, CYP2B-, CYP2E1-, and CYP3A-selective enzyme activities by 15-, 6-, 5-, and 3.4-fold, respectively, the inductions of other enzymes in individual microsomes were lesser. Meanwhile, the fold inductions of all tested CYP enzymes were evenly high in pooled liver microsomes. With pooled liver microsomes, induction folds of 10, 4, 5, and 2.2 were achieved for CYP1A-, 2B-, 2E1- and 3A-selective marker enzymes, respectively, when compared to uninduced control microsomes (Fig. 3). Because of none or little evidences of metabolic pathways of test chemicals or the lack of information on CYP enzymes required to metabolize certain test chemicals, it would be reasonable to use pan CYP-induced microsomes. Therefore, use of pooled liver microsomes would be safer than using uninduced or single protein-enriched microsomes. Moreover, the induction folds of pooled microsomes were much higher than the uninduced microsomes. Therefore, pooled liver microsomes were chosen for the subsequent experiments.

In the present study, specific monooxygenase activities of variously induced, uninduced and pooled liver S-9 fractions were also compared (Supplementary Fig. 1). Comparing to the induced microsomes, as expected, induction folds of CYP enzymes for individually induced or pooled S-9 fractions were very lesser. However, because of none or little evidences of metabolic pathways of test chemicals and lack of information on CYP enzymes required to metabolize certain test chemicals, we decided to use pooled liver S-9 fractions to investigate the β -

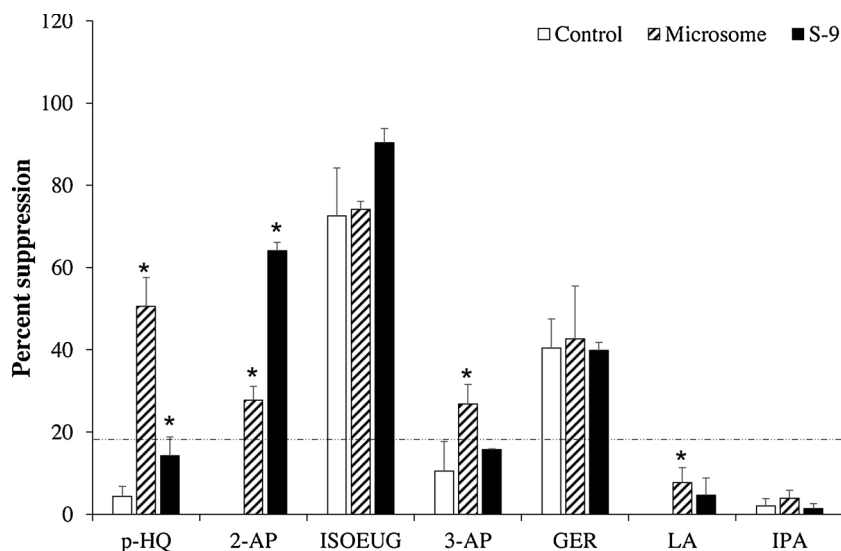


Fig. 2. Effects of rat liver microsomes and S-9 fractions on pre- or pro-haptens-induced suppression of β -galactosidase activity. *E. coli* cells at OD₆₀₀ of 0.5–0.6 (20 μ l) were added into the LB broth medium, pH 7.0, containing IPTG at 30 μ M, and treated with various chemicals at 0.6 mM. Either pooled induced liver microsomes (2 mg/ml), S-9 fraction (2.5 mg/ml), or potassium phosphate buffer, pH 7.4, as control was added into the incubation mixture along with NADPH at 1 mM with a total volume of 500 μ l and incubated at 37 °C for 6 h. β -Galactosidase activity was measured with 2 mg/ml of ONPG prepared in Z-buffer, pH 7.5. Each bar represents the mean percent suppression of β -galactosidase + S.D. of triplicate determinations. p-HQ, p-hydroquinone; 2-AP, 2-aminophenol; ISOEUG, isoeugenol; 3-AP, 3-aminophenol; GER, geraniol; LA, lactic acid; IPA, 2-propanol. The dotted line at 17.3% indicates the cut-off value used in the present study. An asterisk indicates the value significantly different from corresponding control at $P < 0.05$.

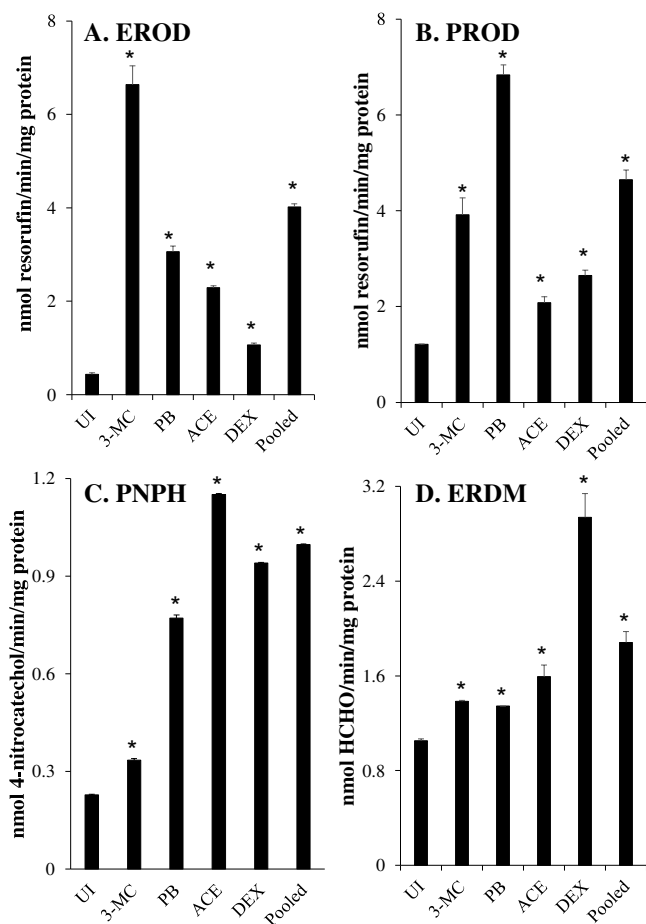


Fig. 3. CYP enzyme activities in induced rat liver microsomes used for the present study. (A) Ethoxyresorufin-O-deethylase (EROD). (B) Pentoxoresorufin-O-depentylase (PROD). (C) p-Nitrophenol hydroxylase (PNPH) (D) Erythromycin N-demethylase (ERDM). Individual microsomes were prepared from male SD rats following treatment with individual inducers as described in the Materials and methods. Each bar represents the mean product produced/min per mg protein + SD of triplicate determinations. UI, un-induced microsome; 3-MC, 3-methylchloranthrene-induced microsome, PB, phenobarbital-induced microsome; ACE, acetone-induced microsome; DEX, dexamethasone-induced microsome; Pooled, mixture of 3-MC-, PB-, ACE- and DEX-induced microsomes in equal volumes. An asterisk indicates the value significantly different from uninduced control at $P < 0.05$.

galactosidase suppression effects of pre- or pro-haptens for comparing the results with microsome-supplemented studies.

As presented in Fig. 4A, the optimum concentrations of liver microsomes were tested for the maximum suppression of β -galactosidase by pre- and pro-haptens after their activation to reactive haptens. *E. coli* cells with optimum cell densities were mixed with LB broth containing 30 μ M of IPTG and various chemicals at 0.6 mM in the presence of various concentrations of microsomes. After mixing, NADPH at 1 mM was added into the tubes to start the reaction at 37 °C. After 6-h of incubation, the samples were lysed to determine β -galactosidase activity. As noted in Fig. 4A, although the results with 1.5 mg/ml of liver microsome were acceptable, the results obtained with 2 mg/ml of liver microsome were better to separate sensitizers and non-sensitizers. Thus, 2 mg/ml was chosen as the optimal concentration of microsomes according to the maximum activation of pre- or pro-haptens to reactive metabolites. Likewise, the optimal incubation time required to achieve the maximum suppression of β -galactosidase activity was determined (Fig. 4B). *E. coli* cells with optimum cell densities were mixed with LB broth containing 30 μ M of IPTG, various chemicals at 0.6 mM along with induced liver microsome at 2 mg/ml and NADPH at 1 mM concentration. The samples were incubated for given time points from 3 h to 24 h. Following respective incubation time, the cells were lysed and β -galactosidase activity was determined. As shown in Fig. 4B, incubation for 6 h at 37 °C would be sufficient to achieve the maximum suppressive effect on β -galactosidase activity. Similarly, the pooled liver S-9 fractions needed for maximum suppression of β -galactosidase by several test chemicals were also determined in the present study (Supplementary Fig. 2). The results indicated that the pooled liver S-9 fraction at 2.5 mg/ml was the best in terms of the suppression of β -galactosidase. It was also observed that, with the increase in the concentration of S-9 fractions, the expression of β -galactosidase by IPTG was lowered, when compared with the microsome-supplemented studies (data not shown).

Subsequently, effects of 4 common solvents on β -galactosidase activity were determined with 6 testing chemicals including 4 pre- or pro-haptens and 2 non-sensitizers in microsome-supplemented system. It was assumed that the vehicles used to dissolve test chemicals would affect CYP enzymes, which would affect the metabolism of test chemicals, and ultimately would affect the suppressive effects on β -galactosidase activity. The solvent affecting enzyme's potency to activate test chemicals might cause less suppression of β -galactosidase by test chemicals. *E. coli* cells with optimum cell densities were mixed with LB broth containing 30 μ M of IPTG, and treated with various chemicals at 0.6 mM that were dissolved in either DMSO, acetonitrile, acetone or

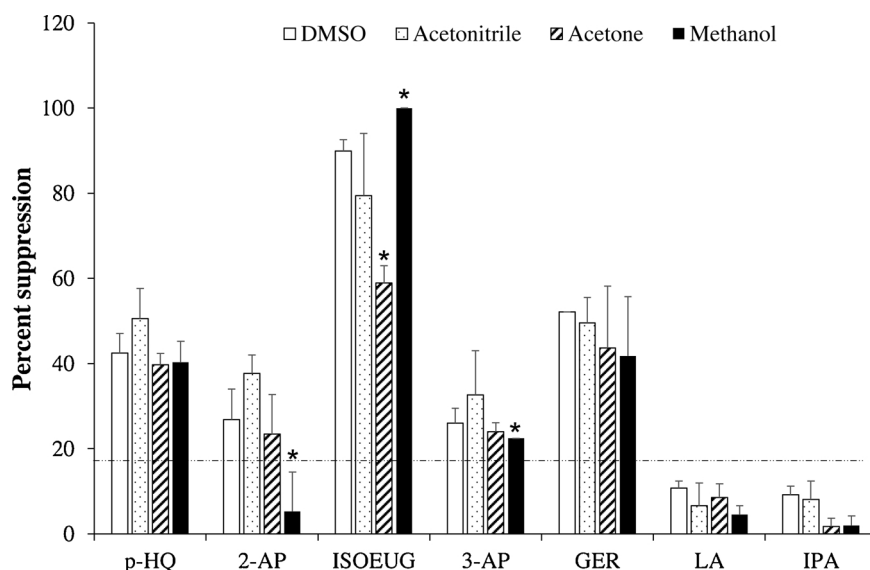
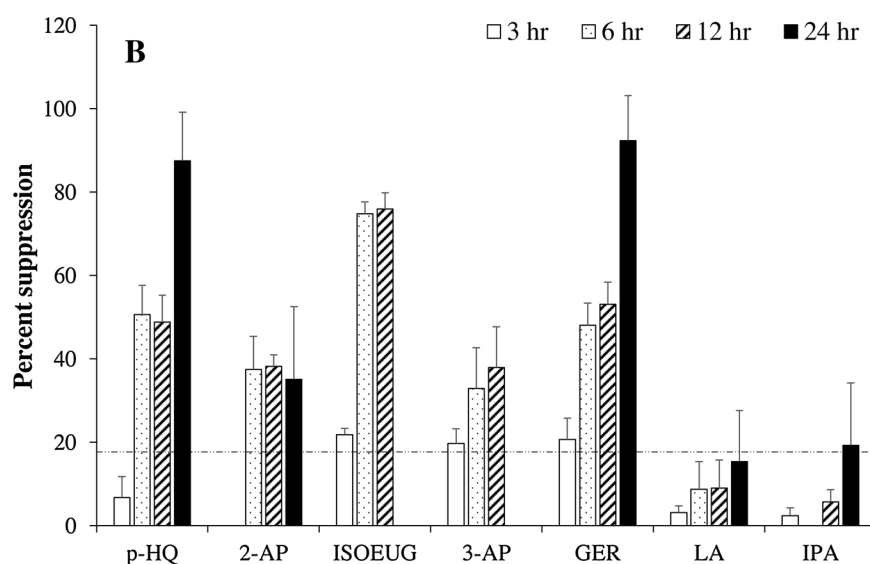
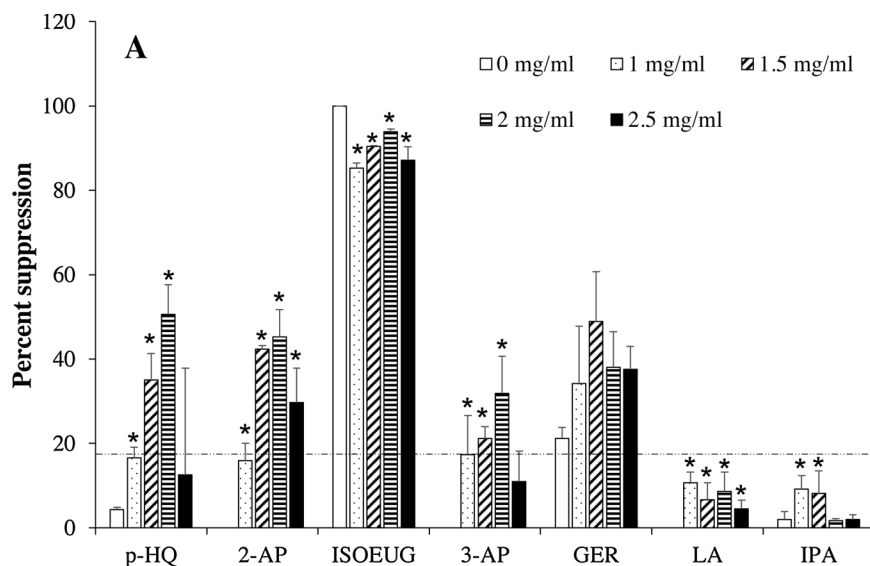


Fig. 4. (A) Effects of liver microsomes on pre- or pro-haptens-induced suppression of β -galactosidase activity. *E. coli* cells at optimum densities were mixed with 30 μ M IPTG in LB broth. Chemicals at 0.6 mM in acetonitrile along with various concentrations of pooled liver microsomes and NADPH at 1 mM were added into the mixture and incubated at 37 °C for 6 h in the total volume of 0.5 ml. Cells were lysed and β -galactosidase activity was determined with 2 mg/ml of ONPG prepared in Z-buffer, pH 7.5. Each bar represents the mean percent suppression of β -galactosidase + S.D. of triplicate determinations. An asterisk indicates the value significantly different from corresponding control at $P < 0.05$. (B) Time-dependent suppression of β -galactosidase induced by pre- or pro-haptens in the presence of 2 mg/ml of pooled liver microsomes. *E. coli* cells at optimum cell densities were mixed with 30 μ M IPTG in LB broth, test chemical at 0.6 mM in acetonitrile, pooled microsomes and NADPH at 1 mM in the total volume of 500 μ l. The mixture was incubated at 37 °C for various time intervals and β -galactosidase enzyme activity was determined. Each bar represents mean percent suppression of β -galactosidase + S.D. of triplicate determinations. p-HQ, p-hydroquinone; 2-AP, 2-aminophenol; ISOEUG, isoeugenol; 3-AP, 3-aminophenol; GER, geraniol; LA, lactic acid; IPA, 2-propanol. The dotted line of 17.3% indicates the cut-off value used in the present study.

Fig. 5. Effects of vehicles on chemical-induced suppression of β -galactosidase activity. *E. coli* cells at optimum cell densities were mixed with 30 μ M IPTG in LB broth and treated with various chemicals at 0.6 mM prepared in various vehicles at 0.4% final concentration. 2 mg/ml of pooled microsomes and 1 mM NADPH was added into the mixture and incubated at 37 °C for 6 h. Following incubation, cells were lysed and β -galactosidase activity was determined with 2 mg/ml ONPG. Each bar represents mean percent suppression of β -galactosidase + S.D. of triplicate determinations. p-HQ, p-hydroquinone; 2-AP, 2-aminophenol; ISOEUG, isoeugenol; 3-AP, 3-aminophenol; GER, geraniol; LA, lactic acid; IPA, 2-propanol. The dotted line of 17.3% indicates the cut-off value used in the present study. An asterisk indicates the value significantly different from DMSO control at $P < 0.05$.

Table 2
Percent suppression of β -galactosidase activity by 31 test chemicals.

No.	Chemicals	LLNA category	Vehicle	Percent suppression of β -galactosidase activity												Judgement
				Microsome-incorporated group						Microsome-unincorporated group						
				0.4 mM	SD	0.6 mM	SD	0.8 mM	SD	0.4 mM	SD	0.6 mM	SD	0.8 mM	SD	
1	p-Hydroquinone	Strong	Acetonitrile	41.5	8.9	50.6	7.0	54.5	11.8	2.7	2.6	4.3	2.5	6.0	3.9	+
2	2-Aminophenol	Strong	Acetonitrile	27.2	19.3	27.7	3.4	42.3	5.0	1.0	1.7	0.0	0.0	0.7	1.2	+
3	p-Phenylenediamine	Strong	Acetonitrile	31.9	10.7	53.7	2.0	45.1	6.6	11.1	17.0	17.2	7.5	3.6	6.3	+
4	Benzo(a)pyrene	Strong	DMSO	29.6	14.4	31.8	11.1	35.5	10.5	0.0	0.0	0.0	0.0	0.7	1.3	+
5	CD3	Strong	DMSO	71.2	12.1	97.7	0.2	97.8	3.7	70.2	33.9	77.4	17.5	80.3	18.5	+
6	Metol	Strong	DMSO	35.5	17.6	59.4	12.1	75.3	10.0	64.8	21.5	50.9	16.8	66.0	4.3	+
7	Lauryl gallate	Strong	DMSO	2.3	2.3	4.8	5.7	19.7	4.4	53.2	19.2	54.5	18.4	68.5	20.4	+
8	Propyl gallate	Strong	Acetonitrile	19.8	10.5	9.2	11.4	11.5	8.1	68.9	15.5	63.9	9.4	58.6	6.3	+
9	Isoeugenol	Moderate	Acetonitrile	50.4	12.5	74.1	2.0	73.0	23.9	43.2	9.0	72.6	11.6	90.7	6.1	+
10	3-Aminophenol	Moderate	Acetonitrile	22.3	10.9	26.8	4.8	30.2	1.8	1.81	15.9	10.5	7.2	3.4	5.9	+
11	1-Naphthol	Moderate	Acetonitrile	53.3	11.1	80.2	3.5	97.7	6.9	76.2	1.4	96.2	1.5	85.2	14.4	+
12	4-Amino-m-cresol	Moderate	DMSO	3.1	5.4	33.4	4.1	22.4	32.7	67.5	10.1	72.9	10.5	70.3	13.0	+
13	Methyl eugenol	Moderate	Acetonitrile	26.0	7.3	29.5	8.0	34.5	3.0	2.2	2.9	19.6	23.8	44.5	4.6	+
14	Farnesol	Moderate	Acetonitrile	20.8	6.7	22.4	2.6	34.4	3.1	38.9	19.8	52.0	9.0	49.9	14.3	+
15	Eugenol	Weak	Acetonitrile	43.6	13.8	56.1	2.3	52.7	16.0	22.9	9.4	36.6	13.8	73.0	11.6	+
16	Benzocaine	Weak	Acetonitrile	34.8	8.1	24.6	2.2	29.1	5.0	22.7	7.7	19.3	5.2	31.7	11.6	+
17	Cinnmyl alcohol	Weak	Acetonitrile	21.7	11.3	30.0	2.0	50.2	10.3	15.0	7.1	22.3	4.9	43.8	9.7	+
18	Aniline	Weak	Acetonitrile	25.0	14.7	26.5	3.6	26.8	3.0	9.0	3.4	11.5	10.8	1.8	3.1	+
19	(R)-(+)-Limonene	Weak	Acetonitrile	0.4	0.8	8.7	4.1	9.2	9.7	10.0	6.9	12.8	5.4	2.8	4.8	-
20	Geraniol	Weak	Acetonitrile	21.8	14.5	42.6	12.9	63.1	19.0	28.3	5.4	40.4	7.1	48.0	8.6	+
21	Methyl salicylate	Non-sensitizer	Acetonitrile	51.8	11.4	61.7	1.4	65.6	15.8	12.1	2.9	13.7	7.2	16.3	2.5	+
22	1-Butanol	Non-sensitizer	Acetonitrile	9.8	6.8	9.2	6.7	15.1	5.7	1.4	2.5	2.2	3.8	18.9	13.5	-
23	Salicylic acid	Non-sensitizer	Acetonitrile	54.9	9.5	60.1	7.9	65.5	11.9	31.0	9.3	36.5	4.9	39.6	9.0	+
24	Lactic acid	Non-sensitizer	Acetonitrile	14.2	4.0	10.7	6.6	13.7	3.8	1.8	3.1	0.0	0.0	10.8	2.3	-
25	Glycerol	Non-sensitizer	DMSO	9.5	5.3	1.0	1.7	19.1	3.3	0.0	0.0	3.2	5.5	5.8	9.6	-
26	2-Propanol	Non-sensitizer	Acetonitrile	3.1	4.3	1.7	2.0	9.2	8.1	0.0	0.0	2.0	1.8	6.4	7.2	-
27	4-Hydroxybenzoic acid	Non-sensitizer	Acetonitrile	23.7	8.1	26.2	3.6	36.6	15.6	5.1	4.1	8.7	6.5	13.6	8.4	+
28	Tween 80	Non-sensitizer	Acetonitrile	12.5	5.5	16.3	2.1	6.5	5.7	8.2	7.5	9.5	8.3	4.8	4.3	-
29	Sulfanilamide	Non-sensitizer	Acetonitrile	1.4	2.4	11.7	2.2	20.0	11.8	2.6	4.6	6.1	6.6	14.3	0.7	-
30	Vanillin	Non-sensitizer	Acetonitrile	4.1	7.1	8.2	14.3	20.7	2.8	2.3	3.9	8.1	8.6	21.0	4.6	-
31	4-Aminobenzoic acid	Non-sensitizer	Acetonitrile	7.6	2.8	5.3	2.8	15.4	5.9	1.2	2.0	4.6	7.9	4.0	3.7	-

Each value represents the mean suppression of β -galactosidase $\pm$ S.D. of triplicate determinations. The percent β -galactosidase suppression of < 0 or > 100 were regarded as 0 and 100%, respectively. When the suppression of β -galactosidase was $\geq 17.3\%$ in either microsome-incorporated group or microsome-unincorporated group at 0.6 mM test chemical, the result was regarded as '+'. The test chemical showing percent suppression of $< 17.3\%$ at 0.6 mM was judged as '-'.

methanol. The final concentration of each vehicle was 0.4%. Pooled liver microsomes at 2 mg/ml and NADPH at 1 mM was added into the samples and incubated at 37 °C for 6 h. As shown in Fig. 5, only marginal differences were observed between the solvents tested except methanol. It seemed that acetonitrile and DMSO showed relative consistent results. Thus, acetonitrile was chosen as the primary solvent in the main study. If the chemicals were not dissolved in acetonitrile, DMSO was used as a second choice. The vehicle used to dissolve each test chemical was listed in Table 2. The same solvent system was chosen for the experiments with pooled liver S-9 fractions.

Following optimization, 31 chemicals, which were considered as the skin sensitizers and non-sensitizers in LLNA studies, were studied for their potential of β -galactosidase suppression (Table 2). The detailed information on test chemicals used were given in Table 1. With optimum cell densities, *E. coli* cells were co-incubated with three individual concentrations of chemicals and incubated at 37 °C for 6 h in presence or absence of pooled induced liver microsomes. Following incubation, the β -galactosidase enzyme activity and percent suppression of β -galactosidase activity by chemicals were obtained. Among 20 pre- or pro-haptens, 8 were strong sensitizers, 6 were moderate sensitizers and 6 were weak sensitizers, based on the categorization by LLNA studies (Gerberick et al., 2007; Natsch and Haupt, 2013; Troutman et al., 2011; Urbisch et al., 2016). Based on our previous study (Nepal et al., 2018a), a cut-off criterion of 17.3% was applied to all chemicals to categorize them as skin sensitizers and non-sensitizers. A decision model was developed in which a positive value in either microsome-used or -unused group would be considered as a sensitizer. All sensitizers except (R)-(+)-limonene showed positive results in either

microsome-used or -unused groups. (R)-(+)-limonene, a major component of citrus fruit peels, which was considered as a weak sensitizer in LLNA studies was only predicted as a false negative in the current study when the cut-off percent of 17.3 was applied at 0.6 mM test chemical, as used in our previous study (Nepal et al., 2018a). Some chemicals, such as propyl gallate and lauryl gallate only suppressed the β -galactosidase activity when no microsome was added. Because they showed positivity in microsome-unused groups, propyl gallate and lauryl gallate were also considered as sensitizers in the present study. Similarly, among 11 non-sensitizers tested, 3 chemicals (i.e., methyl salicylate, salicylic acid, and 4-hydroxybenzoic acid) were falsely categorized as sensitizers after incubation with pooled microsomes, when a cut-off of 17.3% was applied. As shown in Table 3, if the individual prediction of either microsome-used or -used group was considered, chemicals, such as lauryl gallate and propyl gallate, were also considered as non-sensitizers and the accuracy would be lesser because of the effect of wrongly judged chemicals. However, after considering at least one positive as a sensitizer in either microsome-used or -unused group, the sensitivity was improved to 95.0%, 95.0%, and 95.0% with all the chemical concentrations tested (Table 4). Thus, a combination model was chosen as our final decision model rather than individual models (Fig. 6). With the results, the overall sensitivity, specificity and accuracy obtained were 95%, 72.7% and 87.1%, respectively, as summarized in Table 4. The results clearly showed that the incorporation of induced liver microsomes in *E. coli* culture system could improve the predictive capacity.

Similarly, all 31 test chemicals were tested with liver S-9 fraction-supplemented system. As shown in Supplementary Table 1, by using

Table 3

Prediction modeling of either microsome-used or -unused groups at various chemical concentrations.

Chemical concentrations (mM)	Cut-off (%)	Sensitivity (%)		Specificity (%)		Positive predictivity (%)		Negative predictivity (%)		Accuracy (%)	
		W/M	Wo/M	W/M	Wo/M	W/M	Wo/M	W/M	Wo/M	W/M	Wo/M
0.4	17.3	85.0	60.0	72.7	90.9	85.0	92.3	72.7	55.6	80.7	71.0
0.6		85.0	65.0	72.7	90.9	85.0	92.9	72.7	58.8	80.7	74.2
0.8		90.0	65.0	72.7	90.9	85.7	92.9	80.0	58.8	83.9	74.2

W/M, microsome-used group; Wo/M, microsome-unused group.

pooled S-9 fractions, most of the pre- and pro-haptens showed lesser β -galactosidase suppression than by microsomes. Nevertheless, 2-aminophenol showed higher β -galactosidase suppression with S-9 fractions than microsomes. Among 11 non-sensitizers, two non-sensitizers (i.e., salicylic acid and methyl salicylate) were predicted as false positives. 4-Hydroxybenzoic acid, a false positive in microsome-used group, was correctly evaluated as non-sensitizer in S-9 fractions-used group. Moreover, test chemicals, such as CD3, metol, lauryl gallate, propyl gallate, 1-naphthol, 4-amino-m-cresol and methyl eugenol showed lesser β -galactosidase suppression potential. This might be because of the presence of several enzymes in S-9 fraction that might interfere with the chemicals' action to suppress β -galactosidase enzymes. Therefore, with using pooled S-9 fractions, the sensitivity, specificity and accuracy were very less in comparison with microsome-used groups, as shown in Supplementary Table 2. When we apply the same combination model as in the microsome-used group, then the accuracy would be 77.4% at 0.4 mM, 80.6% at 0.6 mM and 74.2% at 0.8 mM chemical concentrations. The results clearly indicate that the predictability would be better with microsomes than S-9 fractions.

4. Discussion

With the motive of developing *in vitro* alternative methods for skin sensitization, several approaches have been made, offering relatively high precision and accuracy. Nonetheless, the methods somewhat lacked the complete *in vivo* scenario of metabolic activation of test chemicals, by which the accurate prediction for pre- or pro-haptens would be limited. To develop a comprehensive *in vitro* method for skin sensitization, a test incorporated with metabolic activation system would be necessary. One of the methods for testing skin sensitization potential of chemicals recently established in our laboratory was to use a bacterial culture expressing β -galactosidase enzyme that can be induced by IPTG (Nepal et al., 2018a). During the induction of β -galactosidase by IPTG treatment, *E. coli* cells would be very vulnerable to the toxic insults by test chemicals due to some reasons. Most importantly, during rapid expression of certain related proteins, β -galactosidase would be affected by reactive chemicals that would be similar with the hapten interaction with proteins in the process of skin sensitization. When we tested 33 chemicals including 22 sensitizers, reasonably high predictive capacity could be obtained with relatively simple test conditions, where neither mammalian cell cultures nor *in vivo* studies are required (Nepal et al., 2018a). In the current study, we incorporated induced rat liver microsomes with this bacterial system to better determine skin sensitization of pre- or pro-haptens that require

metabolic activation.

Although several new elements (i.e., microsomes and NADPH) were added in *E. coli* cultures in the current study, the concentration of *LacZ* gene inducer, IPTG, to stimulate for the maximum production of β -galactosidase enzyme at media pH of 7.0 was observed to be the same as in the previous study (Fig. 1; Nepal et al., 2018a). Although no direct evidences of IPTG-induced toxicities were observed, the concentration of IPTG was maintained as low as possible in order to minimize the chemical burden to the *E. coli* cells. Moreover, some reports have recommended to apply lesser concentrations of IPTG, so that the metabolic burden imposed to cells would be less, and that cells would be free of stresses by IPTG (Dvorak et al., 2015). Therefore, the concentration of IPTG was chosen in such a manner that it would be enough to induce the *LacZ* gene sufficiently, but not to impart negative effects to *E. coli* cells. Likewise, ONPG concentration and pH requirement for the enzyme activity to act optimally was observed to be similar to our previous results (Nepal et al., 2018a).

In the present study, the metabolic capability of uninduced S-9 fractions and microsomes were initially tested for the categorization of pre- and pro-haptens as skin sensitizers in *E. coli* culture system. However, the results with the uninduced fractions were marginal (data not shown). Thereby, induced pooled liver S-9 fractions and microsomes were introduced, because Aroclor 1254-induced S-9 fractions are usually used for Ames test as a metabolic activation system (Irwin et al., 1992). With S-9 fractions, some test chemical, such as 2-aminophenol, showed note-worthy percent suppression of β -galactosidase activity (Fig. 2). Meanwhile, the percent suppression with other chemicals indicated better activation of sensitizers with pooled microsomes than pooled S-9 fractions (Fig. 2). For using pan-CYP-induced metabolic activation system, pooled liver microsome induced by individual inducers were chosen rather than a single protein induced microsome for our study. Parallel to the previous study, the incubation time of *E. coli* with test chemicals for both 6 h and 12 h provided the best results, so that an incubation time of 6-h was fixed for the subsequent experiments (Fig. 4; Nepal et al., 2018a). In addition to CYP enzymes, more additional enzymes might exist in liver S-9 fractions. In this regard, pooled liver S-9 fractions were also tested in the present study, although the CYP enzyme activities were relatively lower than the microsomes (Fig. 3 and Supplementary Fig. 1). As shown in Supplementary Fig. 2, pooled liver S-9 fractions at 2.5 mg/ml would be required for the suppression of β -galactosidase by some test chemicals. Although higher concentrations would give the better results with S-9 fractions, the addition of liver S-9 fractions suppressed the β -galactosidase enzyme production by *E. coli* cells in control groups, which would limit the test

Table 4

Prediction modeling of combination of microsome-used and -unused groups at various chemical concentrations.

Chemical concentrations (mM)	Cut-off (%)	Sensitivity (%)	Specificity (%)	Positive predictivity (%)	Negative predictivity (%)	Accuracy (%)
0.4	17.3	95.0	72.7	86.4	88.9	87.1
0.6		95.0	72.7	86.4	88.9	87.1
0.8		95.0	72.7	86.4	88.9	87.1

The predictability was calculated from the results that the β -galactosidase suppression $\geq 17.3\%$ in either microsome-used or -unused group was regarded as 'positive'.

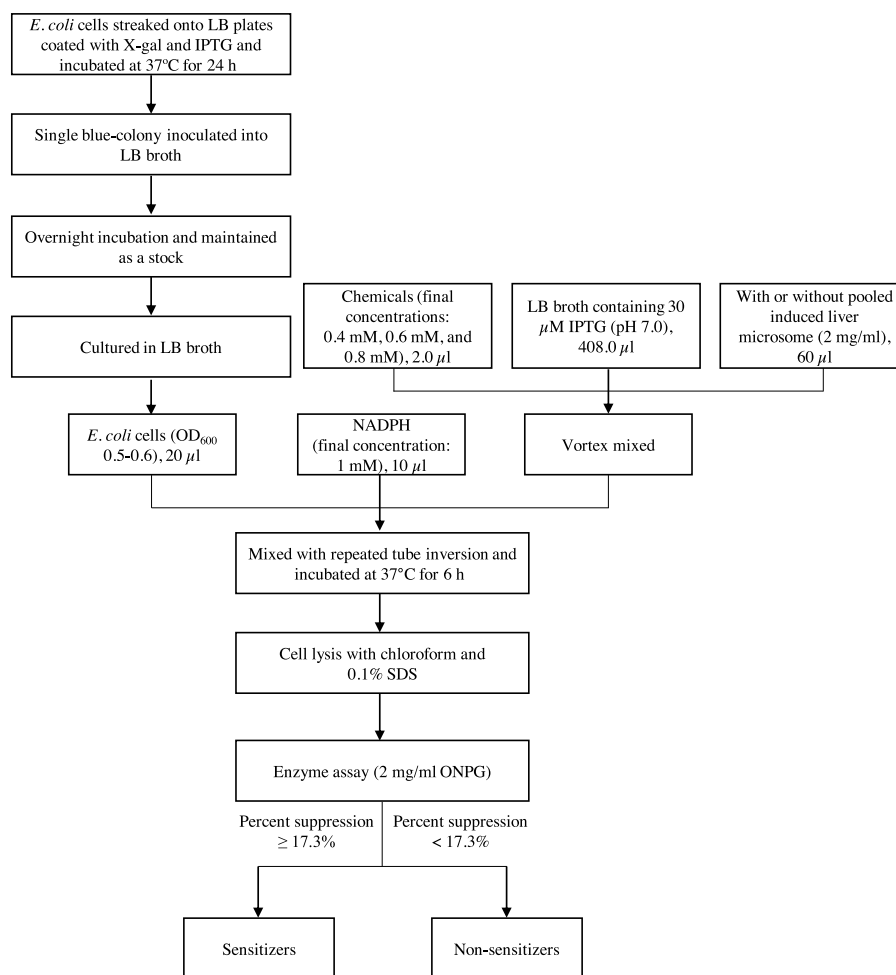


Fig. 6. The proposed classification tree model for the determination of skin-sensitizing potential of test chemicals with test procedures. When the percent suppression of β -galactosidase by a test chemical is $\geq 17.3\%$ in either presence or absence of pooled liver microsomes, the chemical is regarded as 'positive'.

system (data not shown).

Commonly, researchers have used organic solvents as vehicles in cell cultures. For example, a high concentration of DMSO was used to dissolve the chemical to study the metabolic activation of chemicals in a modified KeratinoSens assay (Natsch and Haupt, 2013). However, some reports indicated that various common solvents even in less than 1% concentration inhibit certain CYP enzymes (Chauret et al., 1998; Li et al., 2010). Thus, effects of several solvents for dissolving some pre- or pro-haptens were compared for their activation by pooled microsomes in *E. coli* cultures (Fig. 5). The maximum suppression of β -galactosidase activity by pre- or pro-haptens dissolved in various solvents would referred to have lesser effect on CYP enzymes at 0.4%, so that only marginal difference was observed in the suppression of β -galactosidase in *E. coli* cultures (Fig. 5). Among four solvents tested, methanol showed discrepancy with other solvents for 2-aminophenol. From the results, either DMSO or acetone could also be used for the study, but we chose acetonitrile as the primary solvent of choice for consistency in the present study. For chemicals that were insoluble or difficult to solubilize in acetonitrile, DMSO was chosen as a next choice. The result was consistent with the result from previous reports where acetonitrile was regarded as a solvent of choice in comparison to other solvents in CYP-dependent enzyme studies (Chauret et al., 1998; Li et al., 2010).

Among 22 sensitizers tested in the previous study, six test chemicals were pre- or pro-haptens (Nepal et al., 2018a). Among 6 chemicals, hydroquinone and 2-aminophenol, strong sensitizers, were falsely classified as non-sensitizers due to the lack of metabolic activation (Nepal et al., 2018a). Literatures have shown that peroxidase and

tyrosinase would be required to convert hydroquinone and 2-aminophenol to 1,4-benzoquinone and 2-aminophenoxazin-3-one, respectively (Kettle and Winterbourn, 1992; Washington et al., 2015). Both enzymes would be present in liver cells and assisted to convert those chemicals to their reactive forms (Amanzada et al., 2011; Brown et al., 2001). Although the presence of these enzymes in liver microsomes would be minimal, our present results showed that the incorporation of induced microsomes clearly classified those chemicals as sensitizers, indicating that metabolism by other enzymes to sensitizing metabolite(s) would be possible (Table 2). Moreover, the percent suppression of β -galactosidase was higher with pooled S-9 fractions than with pooled microsomes, suggesting that the enzymes present in cytosolic fraction in S-9 would preferably activate 2-aminophenol (Fig. 2). Among pre- or pro-haptens studied in our previous study, four of them, such as isoeugenol, eugenol, benzocaine, and cinnamaldehyde, were correctly predicted as sensitizers even without any assistance of liver microsomes. The results indicated the likelihood of autoxidation of pre-haptens to active haptens. Furthermore, among several pre- and pro-haptens studied in the present study, most of the chemicals were identified as true positives regardless either with or without the addition of microsomes. However, some chemicals, such as hydroquinone, 2-aminophenol, 3-aminophenol, p-phenylenediamine, aniline, and benzo(a)pyrene studied in the current study, were only able to suppress β -galactosidase activity with the assistance of liver microsomes. The results suggested that the *LacZ* gene expression system in *E. coli* cultures that we developed would be performed in both with and without metabolic activation system as in the Ames test (Flamand et al., 2001).

Interestingly, test chemicals, such as propyl gallate and lauryl gallate gave a reduced response in the presence of microsomes. Conversely, a high percent suppression was achieved in no microsome-added group (Table 2). The results indicated the toxic behavior of parent compounds, but not by their metabolites. These results were also consistent with the results from Booth et al. (1959) and Nakagawa et al. (1995), where metabolic products, such as gallic acid and 4-O-methyl gallic acid, were measured. Because the toxicity of parent compounds, propyl gallate and lauryl gallate, was much higher than their metabolites, this might be a possible reason why lesser or no suppression of β -galactosidase enzyme was observed by propyl gallate or lauryl gallate when incubated with microsomes (Table 2; Nakagawa et al., 1995). Among LLNA-categorized sensitizers tested, limonene, a weak sensitizer in LLNA studies, was detected as false negative in both with and without addition of liver microsomes (Table 2). For the metabolism of limonene, CYP2C9 enzyme was known to be required (Miyazawa et al., 2002). Although further studies are needed, the failure to suppress β -galactosidase by limonene would indicate that the metabolite(s) produced would not have a sensitizing potential. In a pharmacokinetics of D-limonene in a rat model, two major metabolites, dihydroperillic acid and perillic acid, were identified from plasma. Interestingly, perillic acid has been studied as a protective agent against many disorders, such as radiation-induced oxidative stress, cytokine profile, DNA damage, and intestinal toxicity, indicating the non-toxic effects of limonene metabolites (Pratheeshkumar et al., 2010).

Among 20 pre- and pro-haptens tested with S-9 fractions, most of the chemicals showed false negative results (Supplementary Table 3). However, 2-aminophenol showed better result with S-9 fractions than with microsomes. Similar to microsome-used group, lauryl gallate and propyl gallate showed negative results in S-9 fraction-used group. The results would be because of the formation of non-reactive gallic acid and 4-O-methyl gallic acid, respectively. Test chemicals, such as hydroquinone, phenylenediamine, 3-aminophenol, and limonene, could not suppress β -galactosidase enzyme activity in both S-9-used and -unused groups. The results indicated that S-9 fractions could not sufficiently convert them into their reactive forms. Except limonene, all these chemicals showed positive results in microsome-used study.

Among 11 non-sensitizers that were adopted from our previous study, 3 non-sensitizers, such as salicylic acid, methyl salicylate, and 4-hydroxybenzoic acid, showed higher suppressive effects on β -galactosidase in the microsome study (Table 2). In addition, only 2 chemicals, such as salicylic acid and methyl salicylate, showed false positive results in the S-9 study (Supplementary Table 1). In previous study, salicylic acid was considered as the only false positive, which was also consistent with the present results (Table 2). In fact, several conflicting results of salicylic acid were existed in literatures. Salicylic acid, which was considered as a non-sensitizer in several studies (Gerberick et al., 2007), was also categorized as a weak sensitizer and weak skin irritant by some researchers (Arif, 2015; Lee et al., 2014). Moreover, salicylic acid could be hydroxylated to 2,3-dihydrobenzoic acid and 2,5-dihydroxybenzoic acid, and further decarboxylated to 1,2-dihydroxybenzene (catechol) (Stumpf, 2016; Ohmoto et al., 1991). Of interest, catechol was itself considered as a skin sensitizer (Hirose et al., 1999), although the production was not quantitated in the present study. Moreover, from centuries, salicylic acid was used as a skin peeling agent (Arif, 2015). Because of this effect, salicylic acid might showed higher percent suppression of β -galactosidase activity. Similarly, methyl salicylate is a methylated product of salicylic acid. Although the percent suppression of β -galactosidase activity was within the cut-off range of 17.3% in microsome-unused group, with the addition of microsomes, the suppression of β -galactosidase activity was increased to be positive. The results indicated the conversion of methyl salicylate to salicylic acid when acted upon by microsomal enzymes. This hypothesis is further supported by the reports that have stated of easier conversion of methyl salicylate to salicylic acid by blood and liver esterase and could be the reason of methyl salicylate poisoning (Dasgupta and

Wahed, 2014). This further supported the skin sensitizing or irritating behavior of salicylates. 4-Hydroxybenzoic acid also exhibited the similar phenomenon of β -galactosidase suppression similar to methyl salicylate. 4-Hydroxybenzoic acid (p-salicylic acid) which showed no suppressive effect when incubated in the absence of liver microsome (Table 2). However, after the incubation with liver microsomes, the suppression was increased to be falsely positive. This result might indicate the conversion of 4-hydroxybenzoic acid to hydroquinone from the assistance of liver microsome. Moreover, from the same study by Stumpf (2016), 4-hydroxybenzoic acid could be converted to hydroquinone following hydroxylation and decarboxylation. These results might provide a possible evidence that some non-sensitizers might be converted into metabolites that are more potent to suppress β -galactosidase following metabolism by microsomes. Meanwhile, 4-hydroxybenzoic acid, a false positive in microsome-incorporated group, was obtained to be true negative with the S-9 fractions-incorporated group. This result suggested that, with the existence of lesser enzymes in S-9 fractions, the conversion of 4-hydroxybenzoic acid to hydroquinone would be rather limited.

As shown in Table 3, the results of individual prediction modeling with chemicals at 0.4 mM, 0.6 mM, and 0.8 mM would lead to lesser sensitivity of 60.0%, 65.0%, and 65.0%, respectively, in microsome-unused group, and 85.0%, 85.0%, and 90.0%, in microsome-used group. If we consider only the results from either microsome-used group, the chemicals, such as propyl gallate and lauryl gallate, would also be considered as non-sensitizers, and the accuracy would be lesser because of the effect of incorrectly judged chemicals. However, if we consider at least one positive in either microsome-used or -unused group as a sensitizer, the sensitivity would be improved to 95.0%, 95.0%, and 95.0% with the chemical concentrations of 0.4 mM, 0.6 mM, and 0.8 mM, respectively (Table 4). Thus, a combination model in which either one positive result from either microsome-used or -unused group was regarded as a sensitizer would be our final model rather than individual models.

Similar to the microsome, incorporation of S-9 fractions led to lesser individual predictive capacity. However, after applying the combination model as in the microsome-used group, the accuracies were improved in all chemical concentrations. Nevertheless, after observing the sensitivities and specificities at all levels, 0.6 mM chemical concentration would lead to the best result as expected (Supplementary Table 2). In addition, it was clear that the microsome would be the better option to activate pre- or pro-haptens to their reactive metabolites than S-9 fractions. Although the current method could deliver reasonably high predictive capacity, more studies were required to further optimize S-9 fractions and microsomes in the near future. For example, Aroclor 1254-induced S-9 fractions or microsomes will be applied in the test to standardize the metabolic capacity of activation systems, as adopted in the Ames test.

As shown in Supplementary Table 3, the current method was compared with the OECD-adopted methods, such as DPRA, KeratinoSens and h-CLAT, and 2 out of 3 w weight of evidence (WoE) approach (Clouet et al., 2017; Urbisch et al., 2016). We found that test chemicals, such as methyl eugenol, (R)-(+)-limonene, tween 80 and 4-aminobenzoic acid were not studied in all the above *in vitro* methods. In addition, test chemicals, such as metol, isoeugenol, 3-aminophenol, farnesol, aniline, geraniol, and salicylic acid showed variable results in different studies. Because of the insufficient data, a final decision could not be made with test chemicals, such as methyl eugenol, (R)-(+)-limonene, tween 80 and 4-aminobenzoic acid. Meanwhile, other 23 test chemicals were correctly predicted among 27 compared chemicals. In addition, the sensitivity, specificity and accuracy of 31 test chemicals of the current study were compared with the OECD-adopted methods. As shown in Supplementary Table 4, the highest predictivity was obtained with KeratinoSens assay with sensitivity, specificity and accuracy of 89.5%, 90.0%, and 89.7%, respectively. In addition, comparable predictive capacity, such as sensitivity of 88.9%, specificity of 88.9% and

accuracy of 88.9%, were obtained when applying the 2 out of 3 WoE approach with 27 chemicals overlapped. In comparison to other methods, the least accuracy was obtained with DPRA, where several chemicals, such as 3-aminophenol, methyl eugenol, farnesol, aniline, geraniol, and salicylic acid, were incorrectly classified. In the current study, the accuracy was 80.6% and 87.1% when the S-9 fraction and the microsome were used, respectively. In addition, the sensitivity of our current method with microsomes would be the highest among other methods compared. This confirmed that our newly developed method incorporated with induced liver microsomes was as comparable as to the other developed methods. Moreover, the current method would be very advantageous, not only because no mammalian cell or tissue cultures are required for the test, but also because the concentration tested might be lower than the existing methods (Clouet et al., 2017; Urbisch et al., 2016).

Although reasonably high predictivities were achieved in the previous study without a metabolic activation system, the determination of some pre- or pro-haptens were challenging. To overcome the problem, in the present study, we developed a more advanced method in which the chemicals were activated by induced liver microsomes for conversion into their reactive forms to cause skin sensitization. As shown in Table 4, high levels of sensitivity, specificity, and accuracy of 95%, 72.7%, and 87.1%, respectively, were achieved in the combination model with microsomes. The currently developed method can be considered as one of the best methods to determine chemicals as skin sensitizers and non-sensitizers, even though neither mammalian cell cultures nor animals are required in the classification (Fig. 6). Because of the existence of complex molecular events of skin sensitization in animal models, it would be very difficult to mimic the complete *in vivo* scenario of skin sensitization. However, the current new *in vitro* method provided a high prediction capacity in cost-effective and robust manners. Further studies using other strains of bacteria are in progress to verify to what extent the discrepancies in bacterial strain would cause the differences in the results and to further optimize the method to be simpler. In addition, the applicability of current method to detect metallic skin sensitizers are also currently under investigation.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxlet.2019.01.015>.

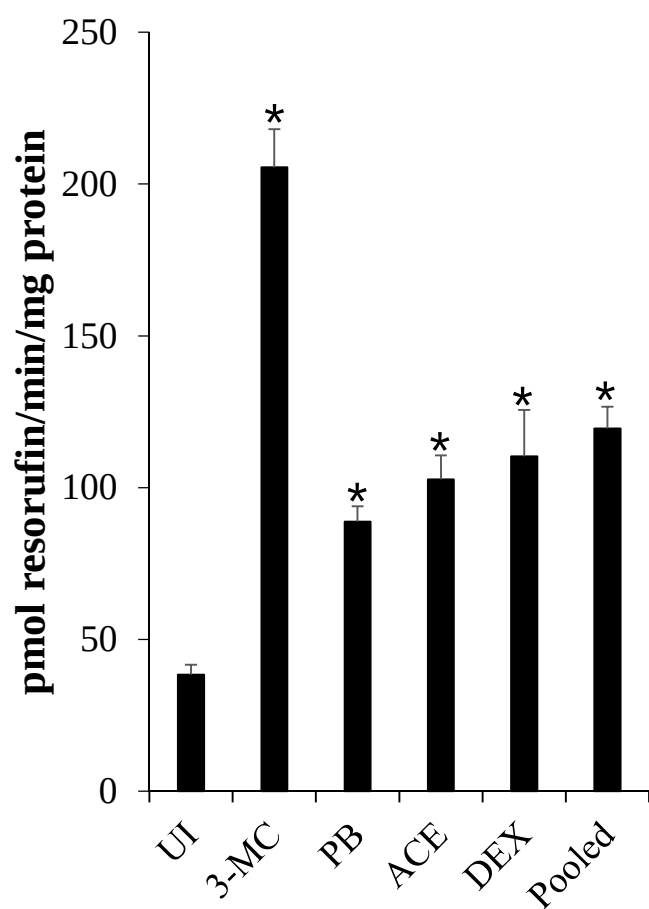
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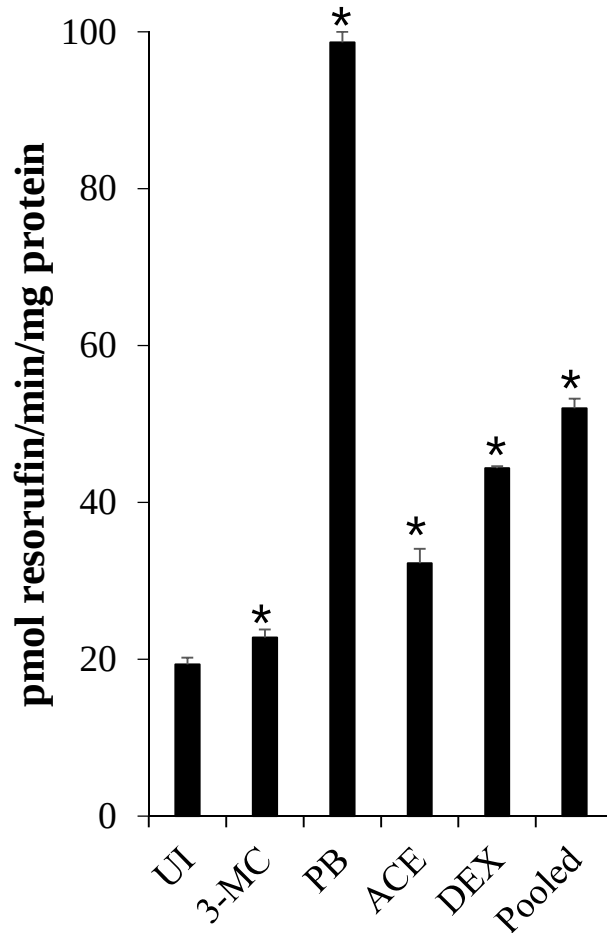
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Supplementary Figure 1.

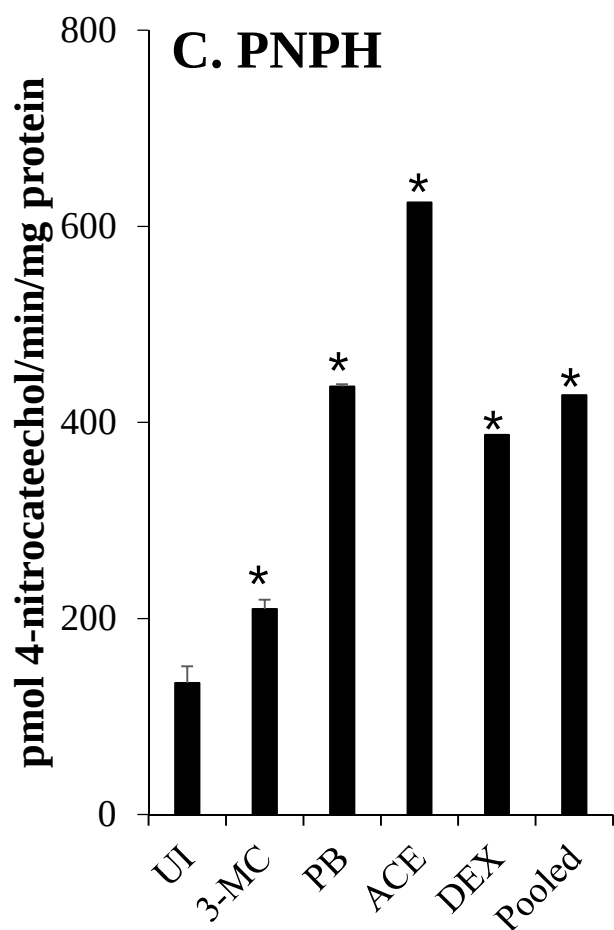
A. EROD



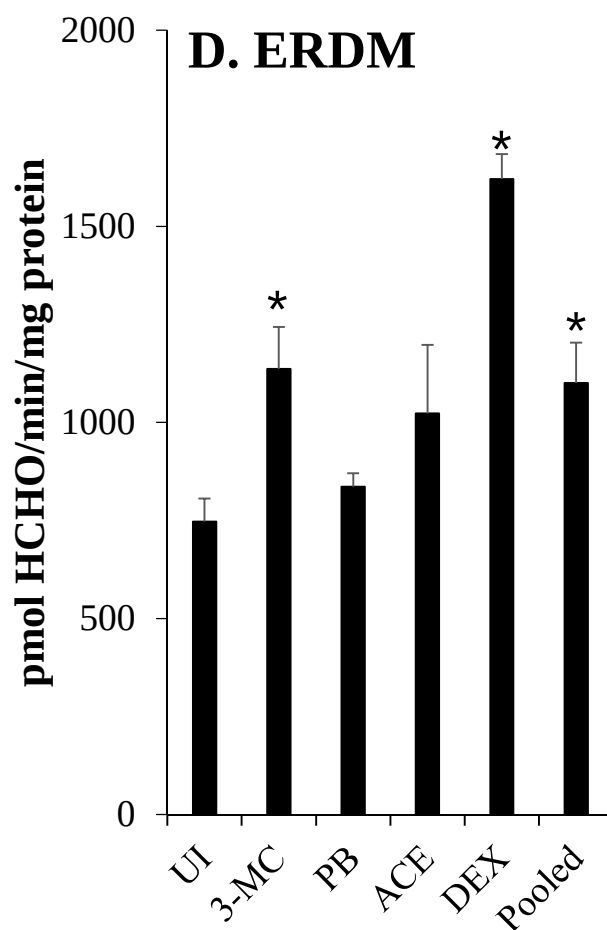
B. PROD



C. PNPH

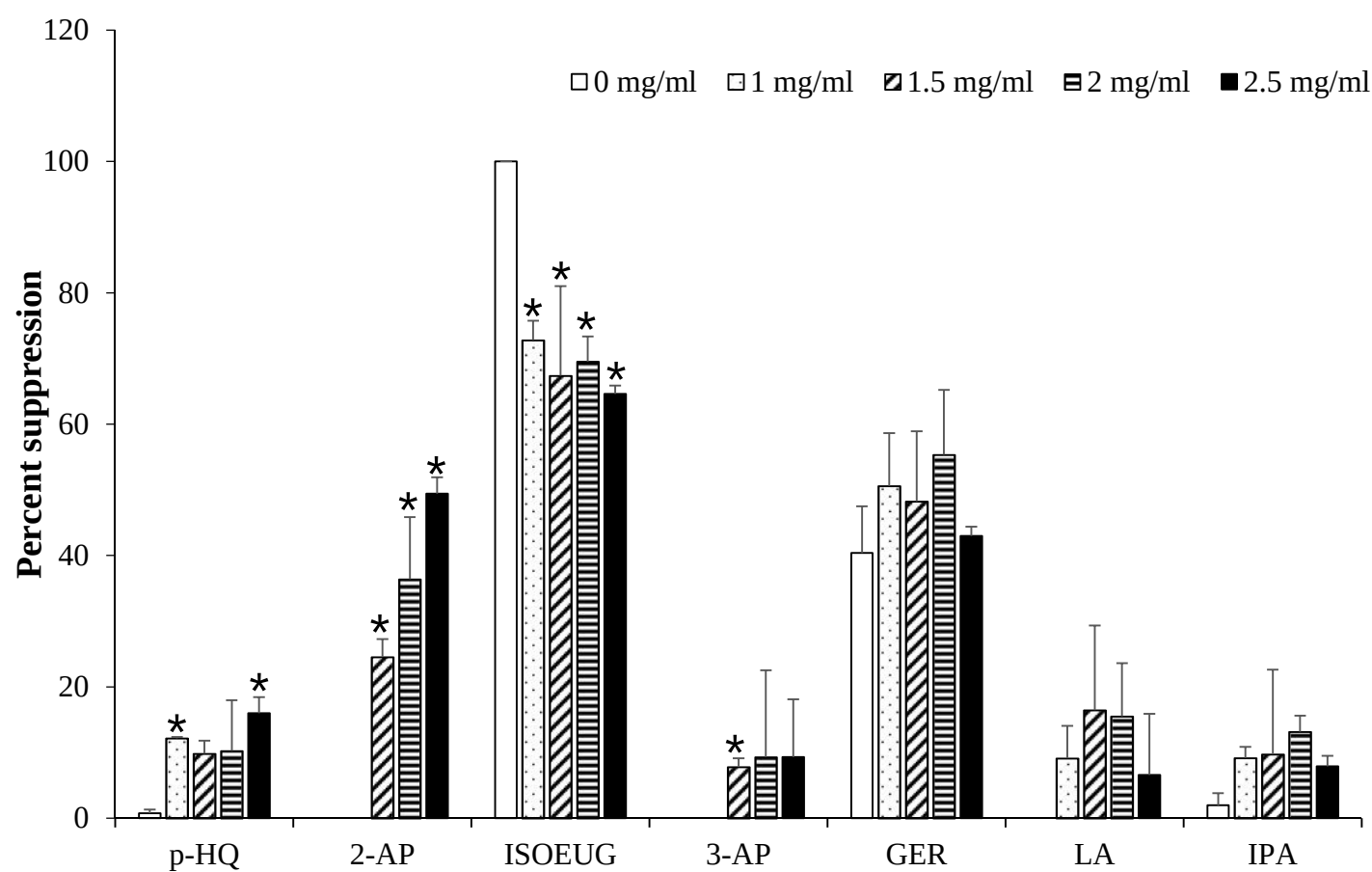


D. ERDM



- Supplementary Fig. 1. CYP enzyme activities in induced rat liver S-9 fractions used for the present study. (A) Ethoxyresorufin-O-deethylase (EROD). (B) Pentoxyresorufin-O-depentylase (PROD). (C) p-Nitrophenol hydroxylase (PNPH) (D) Erythromycin N-demethylase (ERDM). Individual S-9 fractions were prepared from male SD rats following treatment with individual inducers as described in the Materials and Methods. Each bar represents the mean product produced/min per mg protein + SD of triplicate determinations. UI, un-induced S-9; 3-MC, 3-methylchloranthrene-induced S-9, PB, phenobarbital-induced S-9; ACE, acetone-induced S-9; DEX, dexamethasone-induced S-9; Pooled, mixture of 3-MC-, PB-, ACE- and DEX-induced S-9 fractions in equal volumes. An asterisk indicates the value significantly different from uninduced controls at $P < 0.05$.

Supplementary Figure 2.



- Supplementary Fig. 2. Effects of liver S-9 fractions on pre- or pro-haptens-induced suppression of β -galactosidase activity. *E. coli* cells at optimum densities were mixed with 30 μ M IPTG in LB broth. Chemicals at 0.6 mM in acetonitrile along with various concentrations of pooled liver S-9 fractions and NADPH at 1 mM were added into the mixture and incubated at 37°C for 6 hr in the total volume of 0.5 ml. Cells were lysed and β -galactosidase activity was determined with 2 mg/ml of ONPG prepared in Z-buffer, pH 7.5. Each bar represents the mean percent suppression of β -galactosidase + S.D. of triplicate determinations. An asterisk indicates the value significantly different from corresponding controls at $P < 0.05$.

Supplementary Table 1. Percent suppression of β -galactosidase activity by 31 test chemicals in S-9 fraction supplemented test system.

No.	Chemicals	Percent suppression of β-galactosidase activity												Judgement
		S-9 fractions-incorporated group						S-9 fractions-unincorporated group						
		0.4 mM	SD	0.6 mM	SD	0.8 mM	SD	0.4 mM	SD	0.6 mM	SD	0.8 mM	SD	
1	p-Hydroquinone	1.0	1.4	10.3	3.2	22.9	0.9	2.7	2.6	4.3	2.5	6.0	3.9	–
2	2-Aminophenol	24.7	0.7	46.0	2.5	63.1	5.4	1.0	1.7	0.0	0.0	0.7	1.2	+
3	Phenyldiamine	0.0	0.0	9.5	13.5	17.2	1.3	11.1	17.0	17.2	7.5	3.6	6.3	–
4	Benzo(a)pyrene	20.3	1.1	30.5	2.2	42.9	9.2	0.0	0.0	0.0	0.0	0.7	1.3	+
5	CD3	13.1	12.3	25.3	32.6	38.5	8.9	70.2	33.9	77.4	17.5	80.3	18.5	+
6	Metol	16.1	1.2	14.2	11.7	53.6	10.1	64.8	21.5	50.9	16.8	66.0	4.3	+
7	Lauryl gallate	0.0	0.0	0.0	0.0	0.0	0.0	53.2	19.2	54.5	18.4	68.5	20.4	+
8	Propyl gallate	5.6	8.0	9.0	12.7	0.0	0.0	68.9	15.5	63.9	9.4	58.6	6.3	+
9	Isoeugenol	31.4	4.4	64.6	1.3	71.6	8.3	43.2	9.0	72.6	11.6	90.7	6.1	+
10	3-Aminophenol	2.1	3.0	16.6	13.6	9.1	6.9	1.8	15.9	10.5	7.2	3.4	5.9	–
11	1-Naphthol	39.1	1.5	54.9	13.9	76.1	9.1	76.2	1.4	96.2	1.5	85.2	14.4	+
12	4-Amino-m-cresol	0.0	0.0	0.0	0.0	14.3	7.0	67.5	10.1	72.9	10.5	70.3	13.0	+
13	Methyl eugenol	2.7	3.8	19.2	0.0	19.2	26.0	2.2	2.9	19.6	23.8	44.5	4.6	+
14	Farnesol	3.6	5.0	36.1	11.1	37.4	15.1	38.9	19.8	52.0	9.0	49.9	14.3	+
15	Eugenol	0.0	11.5	25.1	3.1	38.6	1.5	22.9	9.4	36.6	13.8	73.0	11.6	+
16	Cinnamyl alcohol	9.5	5.6	11.0	15.5	28.3	2.5	22.7	7.7	19.3	5.2	31.7	11.6	+
17	Benzocaine	3.9	0.0	20.2	8.7	34.0	23.5	15.0	7.1	22.3	4.9	43.8	9.7	+
18	Aniline	0.0	0.0	13.4	10.7	20.4	16.9	9.0	3.4	11.5	10.8	1.8	3.1	+
19	(R)-(+)-Limonene	0.0	0.0	4.6	6.6	6.3	8.9	10.0	6.9	12.8	5.4	2.8	4.8	–
20	Geraniol	23.8	5.4	43.0	1.4	54.6	9.4	28.3	5.4	40.4	7.1	48.0	8.6	+
21	Methyl salicylate	10.0	0.3	27.4	4.8	50.6	21.0	12.1	2.9	13.7	7.2	16.3	2.5	+
22	1-Butanol	9.5	4.3	0.0	0.0	0.0	0.0	1.4	2.5	2.2	3.8	18.9	13.5	–
23	Salicylic acid	31.8	3.9	40.4	5.6	65.1	20.8	31.0	9.3	36.5	4.9	39.6	9.0	+
24	Lactic acid	10.3	1.5	6.6	9.3	8.1	11.5	1.8	3.1	0.0	0.0	10.8	2.3	–
25	Glycerol	0.9	2.0	6.2	8.8	7.7	10.9	0.0	0.0	3.2	5.5	5.8	9.6	–
26	2-Propanol	0.0	0.0	7.9	1.6	4.6	6.5	0.0	0.0	2.0	1.8	6.4	7.2	–
27	4-Hydroxybenzoic acid	13.1	3.4	6.1	4.5	23.0	6.4	5.1	4.1	8.7	6.5	13.6	8.4	–
28	Tween-80	13.2	1.9	4.1	5.8	5.4	7.6	8.2	7.5	9.5	8.3	4.8	4.3	–
29	Sulfanilamide	11.5	5.7	0.6	0.8	1.6	2.2	2.6	4.6	6.1	6.6	14.3	0.7	–
30	Vanillin	5.4	13.3	0.3	0.4	1.2	1.7	2.3	3.9	8.1	8.6	21.0	4.6	–
31	4-Aminobenzoic acid	5.4	2.3	0.0	0.0	0.0	0.0	1.2	2.0	4.6	7.9	4.0	3.7	–

Each value represents the mean suppression of β -galactosidase $\pm$ S.D. of triplicate determinations. The percent β -galactosidase suppression of < 0 or > 100 were regarded as 0 and 100%, respectively. When the suppression of β -galactosidase was $\geq 17.3\%$ in either S-9-incorporated group or S-9-unincorporated group at 0.4 mM, 0.6 mM and 0.8 mM test chemical concentration, the result was regarded as '+'. The test chemical showing percent suppression of $< 17.3\%$ at 0.6 mM was judged as '-'. The results of S-9 fractions-unincorporated groups are the same as in Table 1.

Supplementary Table 2. Prediction modeling of S-9 fractions-used tests at various chemical concentrations.

Chemical concentrations (mM)	Cut-off (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
		With S-9 fractions	With S-9 fractions	With S-9 fractions
0.4 mM	17.3	70.0	90.9	77.4
0.6 mM		80.0	81.8	80.6
0.8 mM		85.0	54.5	74.2

S-9 fractions with 2.5 mg/ml were supplemented in the study. The predictability was calculated from the results that the β -galactosidase suppression $\geq$ 17.3% in either S-9 fraction-used or -unused group was regarded as ‘positive’.

Supplementary Table 3. Comparison of current study model with combination models of DPRA, KeratinoSens, and h-CLAT in a 2 out of 3 weight of evidence approach.

	Chemicals	Current study		DPRA	KeratinoSens	h-CLAT	2 out of 3 approach results (A)	Current study vs. A	References
		S-9 used	Microsome used						
1	p-Hydroquinone	—	+	+	+	+	+	C	Urbisch et al. (2016)
2	2-Aminophenol	+	+	+	+	+	+	C	Urbisch et al. (2016)
3	p-Phenylenediamine	—	+	+	+	+	+	C	Urbisch et al. (2016)
4	Benzo(a)pyrene	+	+	+	+	+	+	C	Urbisch et al. (2016)
5	CD3	+	+	+	+	+	+	C	Natsch et al. (2013)
6	Metol	+	+	+	+	—	+	C	Urbisch et al. (2016), Natsch et al. (2013)
7	Lauryl gallate	+	+	+	+	+	+	C	Urbisch et al. (2016)
8	Propyl gallate	+	+	+	+	+	+	C	Urbisch et al. (2016)
9	Isoeugenol	+	+	+	+	—	+	C	Urbisch et al. (2016)
10	3-Aminophenol	—	+	—	—	+	—	NC	Urbisch et al. (2016)
11	1-Naphthol	+	+	+	+	+	+	C	Natsch et al. (2013)
12	4-Amino-m-cresol	+	+	+	+	+	+	C	Natsch et al. (2013)
13	Methyl eugenol	+	+	—	No data	No data	NA	NA	—
14	Farnesol	+	+	—	+	+	+	C	Urbisch et al. (2016)
15	Eugenol	+	+	+	+	+	+	C	Clouet et al. (2017)
16	Benzocaine	+	+	+	+	+	+	C	Natsch et al. (2013)
17	Cinnmyl alcohol	+	+	+	+	+	+	C	Urbisch et al. (2016)
18	Aniline	+	+	—	—	+	—	NC	Natsch et al. (2013)
19	(R)-(+)-Limonene	—	—	No data	—	+	NA	NA	—
20	Geraniol	+	+	—	+	+	+	C	Natsch et al. (2013)
21	Methyl salicylate	+	+	—	—	—	—	NC	Natsch et al.

								(2013)	
22	1-Butanol	-	-	-	-	-	-	C	Clouet et al. (2017)
23	Salicylic acid	+	+	+	-	+	+	C	Clouet et al. (2017), Natsch et al. (2013)
24	Lactic acid	-	-	-	-	-	-	C	Clouet et al. (2017)
25	Glycerol	-	-	-	-	-	-	C	Clouet et al. (2017)
26	2-Propanol	-	-	-	-	-	-	C	Natsch et al. (2014)
27	4-Hydroxybenzoic acid	-	+	-	-	-	-	NC	Natsch et al. (2014)
28	Tween 80	-	-	No data	No data	No data	NA	NA	-
29	Sulfanilamide	-	-	-	-	-	-	C	Natsch et al. (2014)
30	Vanillin	-	-	-	-	+	-	C	Natsch et al. (2014)
31	4-Aminobenzoic acid	-	-	No data	No data	-	NA	NA	-

True positive, +; true negative, -; NA, not applicable; C, consistent with the former 2 out of 3 approach with the current microsome-used test; NC, not consistent with the former 2 out of 3 approach with the current microsome-used test.

Supplementary Table 4. Prediction modeling of current study model with combination models of DPRA, KeratinoSens, and h-CLAT in a 2 out of 3 weight of evidence approach

	Current study		DPRA	KeratinoSens	h-CLAT	2 out of 3 weight of evidence approach (A)	Current study with microsome-used vs. A
	S-9 used	Microsome used					
Sensitivity (%)	80.0	95.0	73.7	89.5	89.5	88.9	100
Specificity (%)	81.8	72.7	88.9	90.0	80.0	88.9	85.7
Accuracy (%)	80.6	87.1	78.6	89.7	86.2	88.9	95.8