Inhibition Mechanism of UDP-Glucuronosyltransferase 1A6 by Xanthene Food Dyes

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We have reported that erythrosine (ET), a xanthene dye, inhibited uridine 5-diphosphate (UDP)-glucuronosyltransferase 1A6 (UGT1A6). In order to clarify the structure-inhibition relationships of these xanthene dyes, the inhibitory effect of xanthene dye on human UGT1A6 activity was investigated, such as acid red (AR), ET, phloxine (PL), and rose bengal (RB). ET, PL, and RB strongly inhibited human UGT1A6 activity, with IC_{50} values = 0.05, 0.04, and 0.015 mM, respectively. Meanwhile, AR had almost no effect (IC_{50} value = 1.7 mM). ET, PL and RB have four halogen atoms on their xanthene backbone, unlike AR. Meanwhile, some contrast media with high halogens on those aromatic compounds, such as ioxaglic acid, iodixanol, meglumine iotalamate, and diatriazole sodium, did not inhibit human UGT1A6 activity. These results suggest that halogens enhance the inhibitory effect of xanthene dyes. In this study, it was proved that xanthene dyes had an inhibitory effect on human UGT1A6 activity by the combination of xanthene structure and halogens on its. Part of this inhibition by xanthene dyes depends the reaction of $^1O_2$ originated on xanthene dyes by light irradiation, because the inhibition was prevented by $^1O_2$ quenchers, such as NaN_3 and histidine, and in the dark.

Key words ——— uridine 5-diphosphate-glucuronosyltransferase, food colors, rose bengal, erythrosine, xanthene dye, singlet oxygen

INTRODUCTION

Most xenobiotics, such as drugs, non-nutrient substances of low molecular mass in foods, and pollutants, are absorbed and then metabolized by phase I drug-metabolizing enzymes, followed by phase II enzymes, and finally excreted through transporters (phase III enzymes),[1,2] Many drugs are lipophilic and persist in lipophilic membranes composed of lipid matrix. Phase I enzymes convert lipophilic drugs to potentially reactive products and make compounds less toxic,[3] and then phase II drug-metabolizing enzymes conjugate with water-soluble substances, such as UDPGA for uridine 5-diphosphate (UDP)-glucuronosyltransferase (UGT),[4] sulfuric acid for sulfotransferase, and reduced glutathione (GSH) for glutathione *S*-transferase. UGT is the most functional enzyme among the phase II enzymes. We showed that the proximal HNF1 element is essential for the induction of human UGT1A1 by glucocorticoid receptor.[5] Drugs, their metabolites and conjugates with glucuronic acid, sulfate, and glutathione, are excreted by transporters from the liver in bile, from the kidneys in urine and from skin in sweat. The transporters include MDR1 (initially termed P-glycoprotein, now referred to as ABCB1), MRP2 (ABCC2), OATP2, and BSEP. MDR1 functions as a key protein of the blood-brain barrier and prevents anti-cancer drugs from entering into cancer cells during chemotherapy,[6] Dubin-Johnson syndrome, a type of human jaundice, is induced by MRP2 deficiency.[7]

By the development of storage and manufacture methods, processed foods constitute 60% of total foods and are increasing annually. The need for food additives is also increasing.[8,9] These chemical food dyes are also used for coloring cosmetics and pills, as well as foods. Erythrosine (ET) is used as a staining dye for dead *Schizosaccharomyces pombe*[10] and for the investigation of dead bacteria in human den-

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tal caries. During re-evaluation of the safety of these additives, some materials have disappeared. For example, permission to use butter yellow, an azo-dye, was withdrawn due to carcinogenicity within a year after it was granted. Twelve chemical food dyes are permitted by the Japanese Government.[11] There have been some reports showing the inhibition of enzyme activity, such as choline esterase inhibition by ET and sunset yellow, inhibition of sulfation of 17 β-ethinylestradiol by ET,[12,13] and inhibition of dopamine sulfation by tartrazine.[14] The inhibition of some cytochrome P.450 (CYPs) by purpurin and alizarin has also been reported.[15] Meanwhile, amaranth has not been permitted in U.S.A. since 1976 but is permitted in Japan. Most chemical pigments possess anionic sulfate residues that prevent absorption of the pigments in the gastrointestinal tract.[9] Some azo-dyes are reduced by enterobacteria in the intestine and are absorbed in the body.[11] Toxicity studies of these pigments in humans are difficult for many reasons, thus, toxicity studies depend on experimental results in animals.[16]

Meanwhile, phenyl-xanthene dyes, such as rose bengal (RB), ET, phloxine (PL), eosin (ES), uranine (UR), rhodamine (RM), and fluorescein are known as light- enhancing reagents (catalytic light reaction) by the generation of $^1O_2$ on those dyes.[17–23] There are two types of reaction: the first is that drug energy enhanced by light is transferred to biomolecules and free radicals originate on the molecules. The second is that energy is transferred to oxygen, which changes to $^1O_2$. This reaction depends upon the number of halogens on xanthene dyes and the light strength. There are some papers of inactivation of enzymes by xanthene dyes. Na,K-ATPase was inactivated by light in the presence of RB.[23,24] Acetylcholineesterase and some microorganims, such as *Escherichia coli*, *Staphylococcus aureus*, and influenza virus, are inactivated,[17,18,22,25] therefore, it is possible that xanthene dyes inactivate drug-metabolizing enzymes.[26]

In the previous study, we clarified that RB inhibited the activity of UGT1A6 and CYP2A6 by mixed-type inhibitory mechanism.[26] In this study, we clarified the structure-function-relationship of xanthene dyes and also suggested that inhibition depended upon light reaction by the generation of $^1O_2$ on these dyes.

MATERIALS AND METHODS

Dyes ——— The chemical food dyes used were erythrosine B (Food Red No. 3), acid red (Food Red No. 106), phloxine (Food Red No.104), rose bengal (Food Red no. 105) as shown in Fig. 1. These were generously supplied by San-Eigen Co. Ltd. (Osaka, Japan) and passed official approval for purity and safety by the Japanese Government. Non-food additive xanthene dyes used were UR, ES Y, eosin-5-isothiocyanate (E5ic), RM B and xanthene (XT). The contrast media and analog used were: ioxaglic acid (Tanabe Pharmaceutical Co. Ltd., Osaka, Japan), iodixanol (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan), meglumine iotalamate (Daiichi Pharmaceutical Co. Ltd.) and sodium diatrizoate (Amersham Bioscience, Uppsala, Sweden). These compounds contain high halogens in the molecules. These dyes, except E5ic and XT, and contrast media were dissolved in water. E5ic and XT were dissolved in ethylalcohol and methylalcohol, respectively. NaN_3 and histidine were dissolved in water. β-Carotene (Merck) was dissolved in dimethyl sulfoxide (DMSO). Those solutions were used at various appropriate concentrations.

Enzymes ——— Pooled human liver microsomes obtained from Gentests were stored at –80°C. Protein concentration was determined by the standard Bradford method.

Microassay of UGT Activity ——— The microassay method of UGT activity in this study was carried out according to a previous report[4] as follows: The reaction mixture of 50 μl contained 2 mM [^{14}C]UDPGA (0.02 nCi/nmol, a product of American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A.), ligand [*p*-nitrophenol (*p*-NP) or androsterone] and dyes as inhibitors at various concentrations in 20 mM Tris-maleate (pH 7.5) and 1 mM $MgCl_2$. The reactions were started by the addition of human liver microsomes (20–24 μg protein) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 μl ethanol. The mixture was centrifuged at 8000 $\times$ *g* for 5 min and the supernatant was concentrated by evaporation under a vacuum. The residual material was dissolved in 70% ethanol and spotted onto silica plates (Merck silica gel 60, Merck KGaA, Darmstadt, Germany) for thin layer chromatography. The TLC plate was developed in *n*-butanol : dH_2O : acetone : glacial acetic acid : 30% ammonium = 70 : 60 : 50 : 18 : 1.5. The radioactivity of the [^{14}C] products on the thin layer was quantitatively analyzed using the radio-image analyzer Fuji

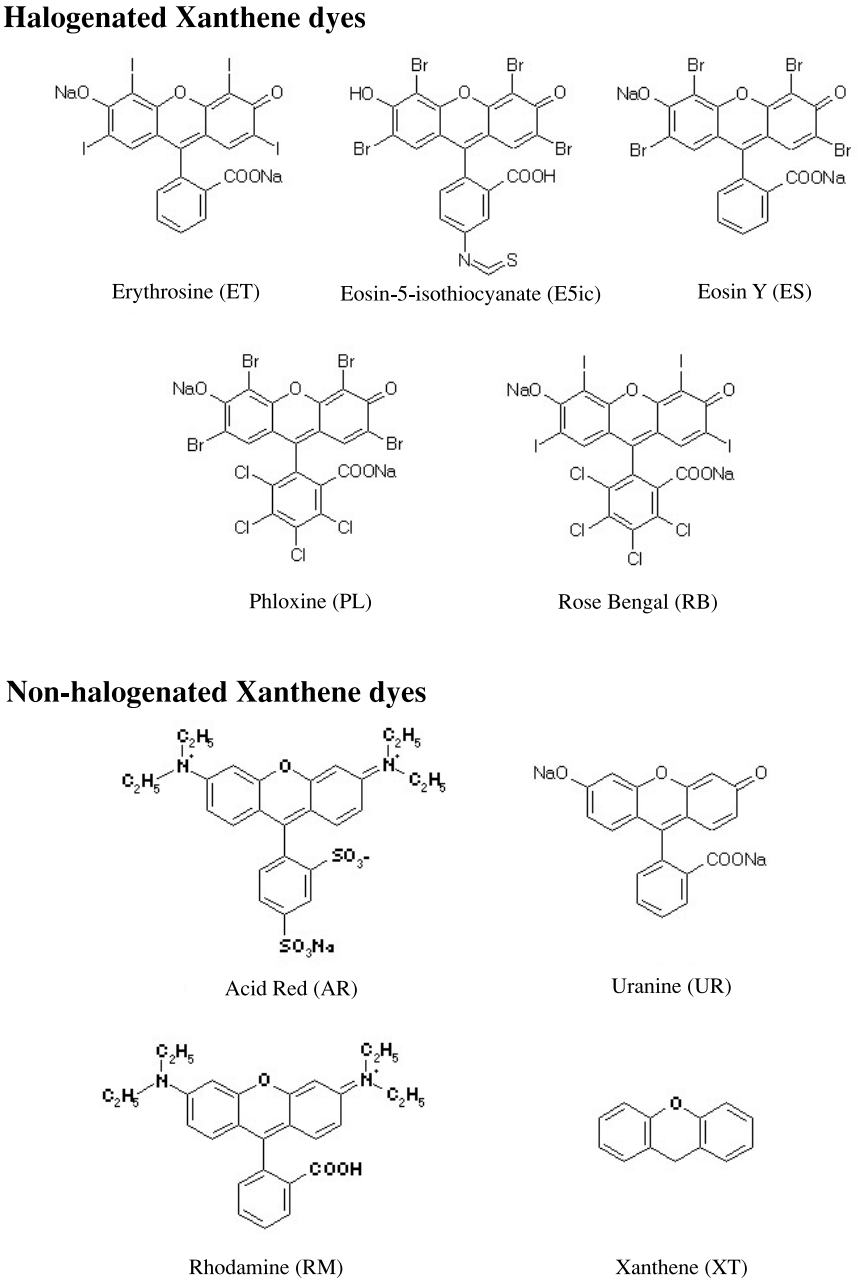


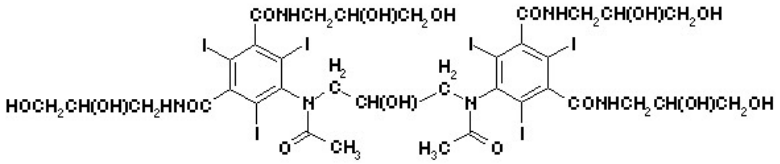
Fig. 1. Chemical Structure of Halogenated Xanthene Dyes, Non-Halogenated Xanthene Dyes and Contrast Media

BAS2500 (Fuji Film, Tokyo, Japan). The [^{14}C] conjugates were found in the upper positions on the thin layer and UDPGA was found in the lower positions (Kanou *et al.,* 2002). Kinetic parameters were calculated from the amount of radioactivity of the conjugates. *p*-NP (for UGT1A6) were used as positive substrates for the UGT reaction. The specific activity of UGT1A6 for *p*-NP in pooled human liver microsomes was 25 nmol/min/mg. General reaction was done under 100 W light at a distance of 2 m (approximately 500 Lux). For the control experiment, the experiments were carried out in the dark (yellow light).

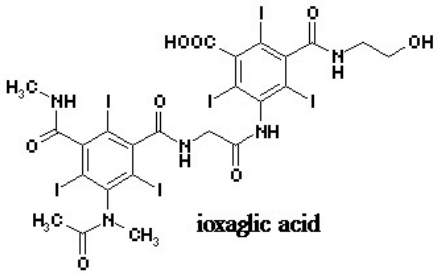
The Reaction to Measure UGT Activity in D_2O ——— For the reaction in D_2O (Merck), substrates and inhibitors were dissolved in D_2O. Tris-maleate (1 M) buffer containing 50 mM $MgCl_2$ was diluted with D_2O to one-fiftieth (2%) and liver microsomes (1 μl) were added to the reaction mixture (49 μl). Thus, the glucuronidation reaction proceeded in 95% D_2O solution.

Statistical Analyses ——— The mean ± S.D. value of each point was calculated from 3 determinations. The validity of the inhibition was examined by

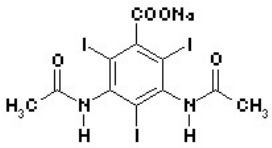
Contrast media



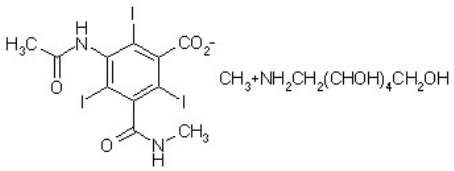
iodixanol



ioxaglic acid



sodium diatrizoate



meglumine iotalamate

Fig. 1. Continued

Student's *t*-test for differences in the presence (control) and absence of an inhibitor. Significant values at the 5% level were taken as effective. * and ** show significant from control ($p < 0.05$ and $p < 0.01$), respectively.

RESULTS

In the previous paper, we showed the inhibition of UGT1A6, UGT2B7 and CYP2A6 by ET among 12 kinds of food additive dyes permitted by the Japanese Government.[26] ET is a xanthene dyes and is highly halogenated. Therefore, we studied the inhibition of UGT1A6 by other two halogenated xanthene dyes, such as RB and PL, permitted food additive dyes, and ES and E5ic, non-permitted dyes, whose structures are shown in Fig. 1. These dyes inhibited UGT activity well as shown in Fig. 2, and these IC_{50} values of ET, RB, PL, ES, E5ic were 0.07, 0.015, 0.04, 0.12, 0.07 mM, respectively. At a concentration of 0.5 mM, the dyes almostly totally inhibited glucuronidation activity. Meanwhile, non-halogenated xanthene dyes, such as acid red (AR), RM, UR and XT, did not inhibited the activity of UGT1A6 as shown in Fig. 3. These IC_{50} values were higher than 1 mM. From these results, we considered that halogenated-aromatic compounds should inhibit UGT1A6 activity, and next we studied inhibition by high-halogenated compounds, such as contrast media, as shown in Fig. 1. However, we found no inhibition using these high-halogenated compounds, such as ioxaglic acid, iodixanol, meglumine iotalamate for contrast media, and sodium diatrizoate for leucocyte preparation.

From these results, we considered that the haloganated xanthene backbone is a key structure and iodine is the most potent element among halogens, bacause RB containing iodine is more potent than PL containing bromine. It is possible that the resonating double bond continuing from a carbonyl bond on the xanthene backbone is essential, as well as halogens on the xanthene backbone itself, in comparing structures in Fig. 1 and inhibition patterns in Figs. 2 and 3. Phenyl residues on xanthene dyes may be another important residue as well as halogens on phenyl residues.

The inhibition of UGT by xanthene dyes was confirmed as a mixed-type mechanism from the pattern by Lineweaver-Burk plots as in the previous

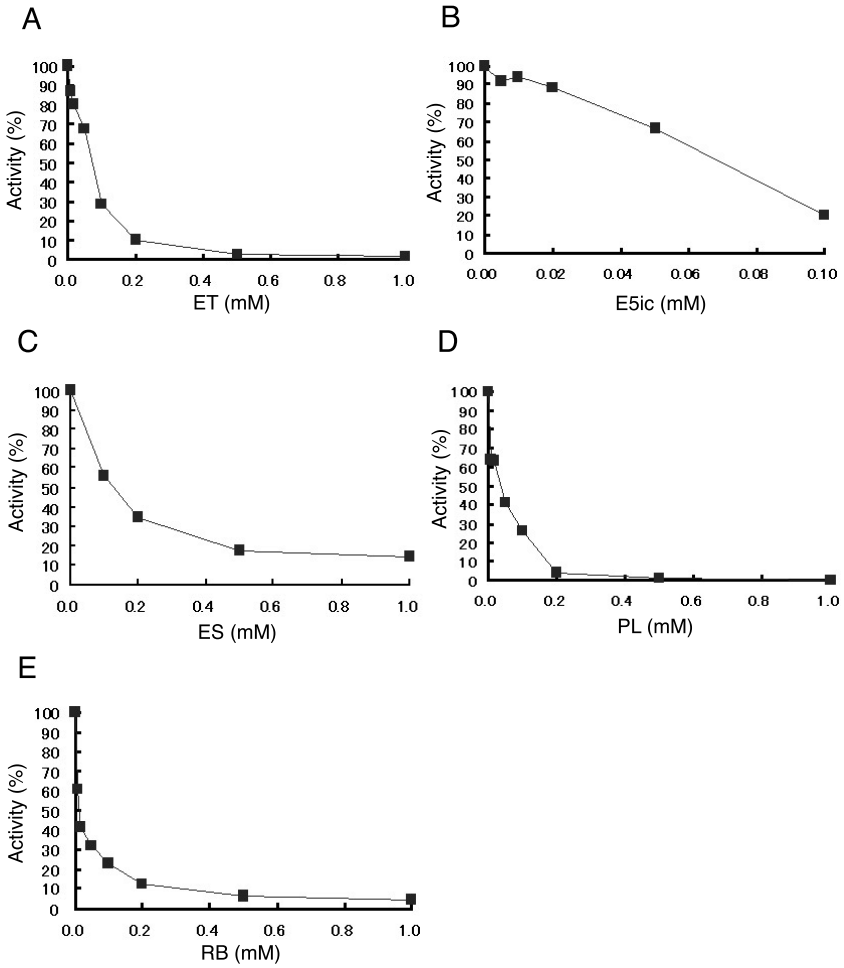


Fig. 2. Effect of Halogenated Xanthene Dyes on Glucuronidation of *p*-Nitrophenol by Human UGT1A6
A, ET; B, E5ic; C, ES; D, PL; E, RB.

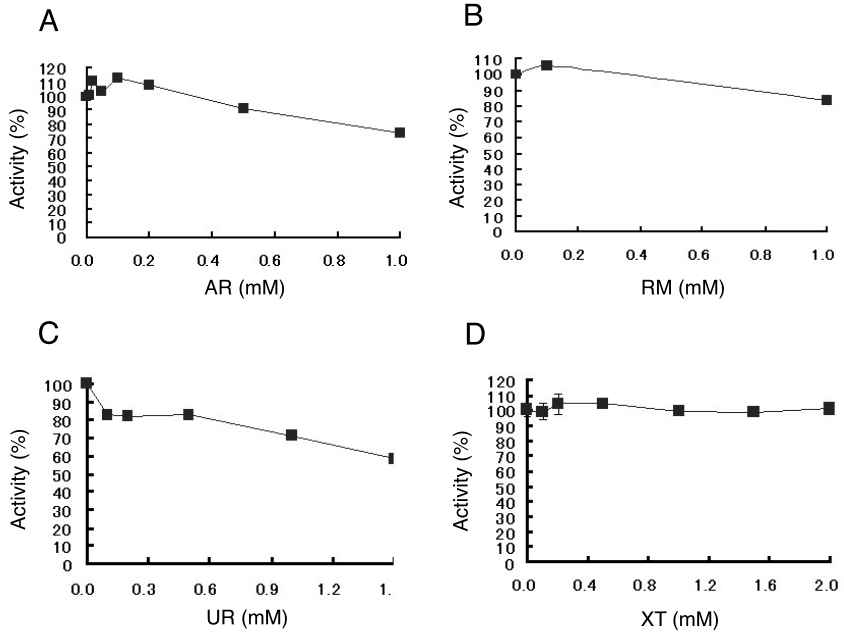


Fig. 3. Effect of Non-Halogenated Xanthene Dyes on Glucuronidation of *p*-Nitrophenol by hUGT1A6
A, AR; B, RM; C, UR; D, XT.

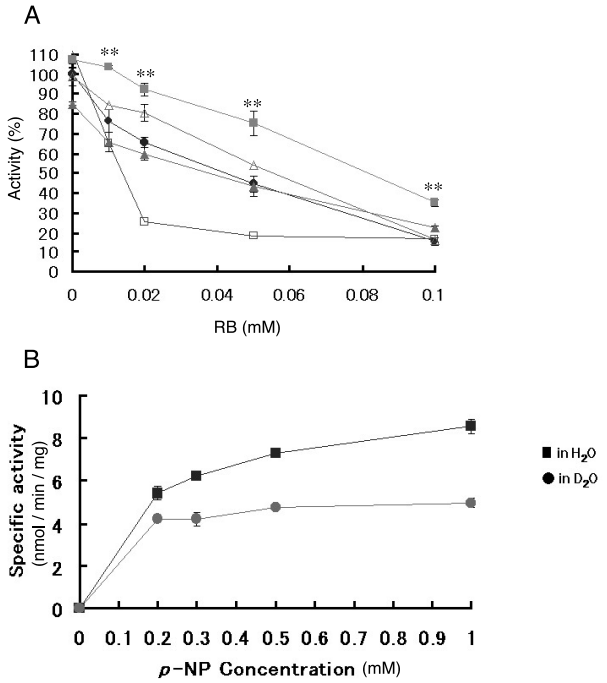


Fig. 4. Effect of Some Quenchers on Glucuronidation Inhibition of *p*-Nitrophenol by RB

In A, the reaction was carried out in 5 mM NaN_3 (■), 5 mM L-Histidine (△), 0.2 mM *β*-carotene (□), 95% D_2O (▲) and in their absence (●). **, $p < 0.01$. In B, the specific activities of hUGT1A6 in D_2O or H_2O are shown.

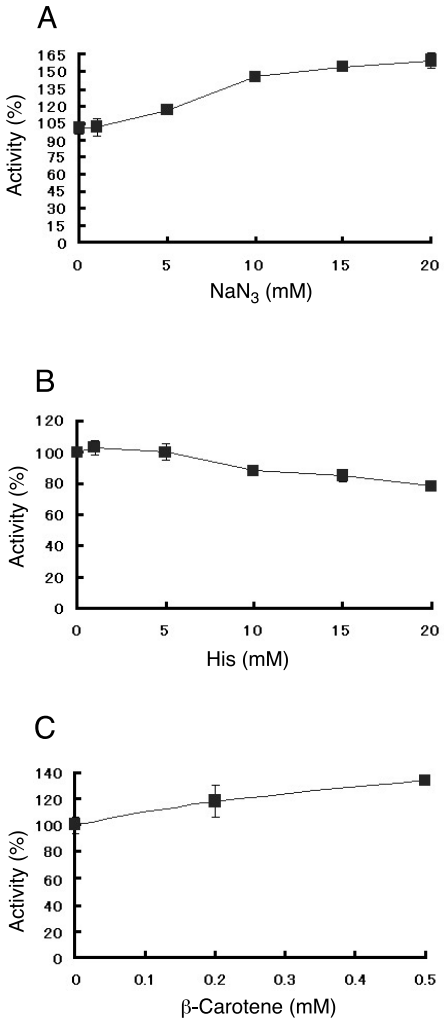


Fig. 5. Effect of $^1O_2$ Quenchers on Glucuronidation of *p*-Nitrophenol by hUGT1A6

A, NaN3; B, L-histidine; C, *β*-carotene.

report.[26] This indicates that inhibition is dependent upon a complexed-type reaction involving enzyme inactivation. In order to clarify the mechanisms, we studied the influence of $^1O_2$ quenchers, such as NaN_3, histidine, and *β*-carotene on glucuronidation inhibition by RB. We also investigated the influence of D_2O on the glucuronidation reaction.

Figure 4A shows that NaN_3 and hisitidine significantly prevented the inhibition by RB, but *β*-carotene did not. NaN_3 and hisitidine are soluble in the reaction mixture but *β*-carotene is insoluble, so we could not obtain clear results with *β*-carotene. The prevention of RB inhibition by NaN_3 and histidine suggests that a part of the inhibition by RB depend upon $^1O_2$ originated on RB molecules activated by light. Fig.4A also shows the increase of inhibition by RB in D_2O solution. Fig. 4B shows the comparison of the activity in water and D_2O, and the result shows that the activity in D_2O is approximately half of the activity in H_2O. This may be because part of this decrease (increase of inhibition by RB) in activity depends upon the long existence (slow disappearance) of $^1O_2$ in D_2O solution, as well as the slightly higher viscosity of D_2O solution. Figure 5 shows that these quenchers (NaN_3, hisitidine, and *β*-carotene) themselves showed no inhibition of glucuronidation of *p*-NP by UGT1A6 in the range of concentration from 1 to 20 mM of NaN_3 and histidine, and 0.2–0.5 mM of *β*-carotene.

Figure 6 indicates the influence of light on RB inhibition. This experiment was carried out at 0.3 mM *p*-NP. We found a significant difference between the values of activity in the dark and light at low concentrations, 0.01, 0.02, and 0.05 mM of RB. This result suggests that the weak inhibition by RB in the dark may depend on the low generation of $^1O_2$ in the dark. We could not find a significant difference at a high concentration, 0.1 mM, of RB. This inhibition in the dark at 0.1 mM RB indicates that this inhibition depends on not only $^1O_2$ but also unknown factors.

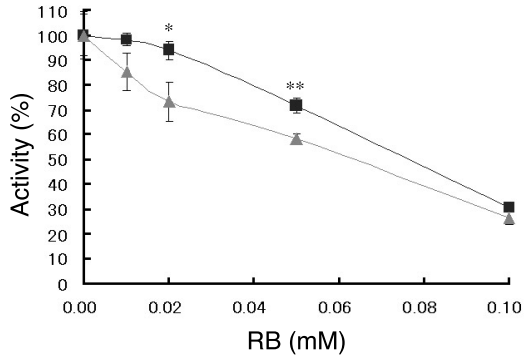


Fig. 6. Comparison of the Inhibitory Effect of RB in the Dark (■) or Light (▲)
*, $p < 0.05$ and **, $p < 0.01$.

DISCUSSION

Chemical food additive dyes are large molecular masses having a strong anionic charge of sulfate or cationic charge on the molecule to prevent absorption in the gastrointestinal tract. It has been described that a few parts of those pigments are absorbed (Japan's Specifications and Standards for Food Additives 1999). Approximately 2 mg total pigments/day is ingested and the concentration in the body is estimated to be 2 nM. This level is lower ($1/10^5$) than the IC_{50} value of RB (0.015 mM), PL (0.04 mM) and ET (0.05 mM) for UGT and the IC_{50} value of Indigo Carmine (0.05 mM) for CYP2A6. Thus, these dyes should not influence on drug metabolism and the inhibition under normal conditions in our body. However, it is necessary for some patients having ulcer in gut to take care of ingestion of chemical food dyes. It is also possible that some cosmetics containing red xanthene dyes, activated by light, may be absorbed in body through skin. Thus, it is recommended for someone who having inflammation on face not to use cosmetics. Recently, the inhibition of troglitazone (oral antidiabetic agent) on glucuronidation catalyzed by human UGT1A6 [27] has been reported. The IC_{50} value of troglitazone is 0.028 mM. The mechanism of troglitazone is not clear, and there is no halogen or resonating conjugated bond in troglitazone.

It has been reported that xanthene dyes generate $^1O_2$ in the light.[17–22] The inactivation of enzymes by xanthene dyes may well proceed in aerobic conditions through type II mechanisms of $^1O_2$ generation. It was reported that $^1O_2$ generation on xanthene dyes is RB > ET > PL > ES >> UR.[18] By this experiment, the strength of inhibition is RB > PL > ET > E5ic > ES >> AR, RM, and UR. This order of inhibition is similar to $^1O_2$ generation. The study of $^1O_2$ quenchers also supports that inhibition by RB is prevented by quenchers, such as NaN_3 and histidine as shown in Fig. 4A. The influence by β-carotene of another quencher was not clear. This may come from insolubility of β-carotene and the study with β-carotene must be done in future. In D_2O, inhibition by RB was promoted, suggesting the $^1O_2$ played the role of inhibition function, because the 16-times long reservation of $^1O_2$ in D_2O solution.[24] However, the quencher results were not complete but partial effects. This may be because UGT1A6 is not a soluble but a membrane-bound enzyme and is buried in lipid bilayers which protects UGT1A6 from $^1O_2$ attack.

There are few studies available for phase 1 and phase II drug-metabolizing enzymes involving chemical food dyes. Many studies were carried out on the toxicity and carcinogenicity of chemical food dyes.[8,14] Our previous results showed that the activity levels of CYP2A6 and UGT in bovine liver microsomes were similar to human liver microsomes[4,28,29] but differed from rat microsomes, as rat microsomes did not involve CYP2A6 activity. Thus, it was considered that bovine microsome data were very similar to human microsome data.

From the structure-function relationships in glucuronidation inhibition, it is very interesting that halogenated xanthene dyes, such as ET, RB, and PL, have inhibitory activity against UGT. Meanwhile, non-halogenated xanthene dyes, such as AR, UR, RM and XT do not possess this inhibitiory activity. ET has anionic phenol residues and AR has cationic ammonium residues, as well as halogenated residues. Other high-halogenated aromatic compounds of contrast media did not show any inhibition of UGT1A6; therefore, halogenated, resonating, aromatic xanthene compounds may be in conditions for $^1O_2$ generation to inhibit enzymes.

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