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Synthesis of novel benzbromarone derivatives designed to avoid metabolic activation

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Keywords

Benzbromarone; metabolic activation; hepatotoxicity; URAT1

Abbreviations

BBR, benzbromarone; CYP, cytochrome P450; DBBQ, 2,6-dibromo-*p*-benzoquinone; DBH, 2,6-dibromohydroquinone; DEX, dexamethasone; hURAT1, human uric acid transporter I; IDR, idiosyncratic adverse drug reactions; LDH, lactate dehydrogenase; MPT, mitochondrial permeability transition

Abstract

We synthesized six novel BBR derivatives that were designed to avoid metabolic activation via *ipso*-substitution and evaluated for their degree of toxicity and hURAT1 inhibition. It was found that all of the derivatives demonstrate lower cytotoxicity in mouse hepatocytes and lower levels of metabolic activation than BBR, while maintaining their inhibitory activity toward the uric acid transporter. We propose that these derivatives could serve as effective uricosuric agents that have much better safety profiles than BBR.

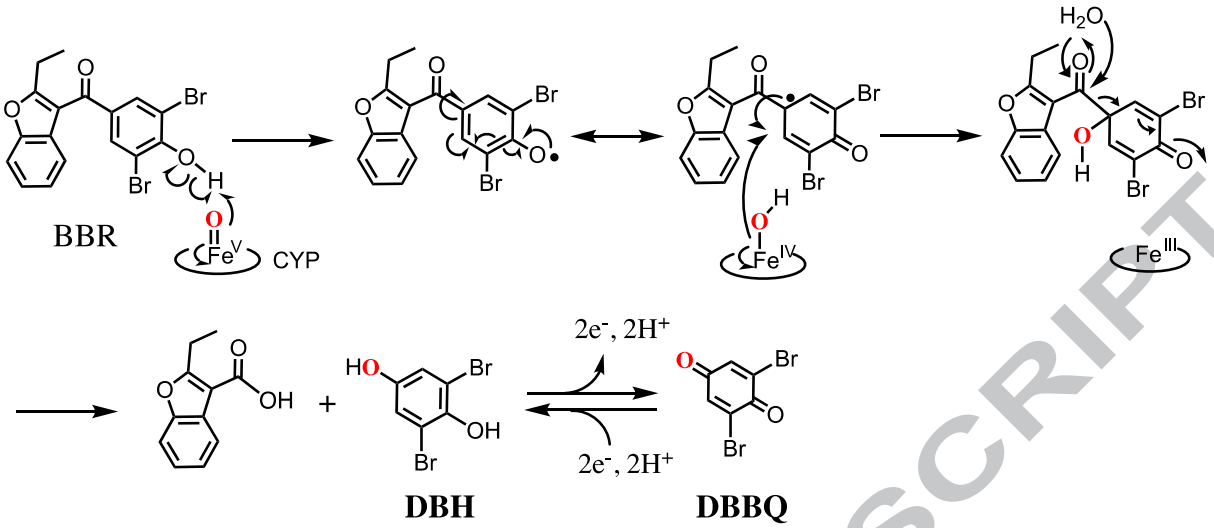
Adverse drug reactions are one of the most important issues that occur during drug development and the clinical usage of drugs in the postapproval stage. Specifically, idiosyncratic adverse drug reactions (IDR) with often serious toxicities occur only in a small percentage of the population and do not show any apparent dose dependency. IDR is a great problem in terms of safety due to its unpredictability, which presents challenges during drug development and leads to postmarketing drug withdrawals and black box warnings.[1] Although there are several hypotheses regarding the mechanisms that underlie IDR, it is widely accepted that IDR is often associated with bioactivation that is mediated by metabolic enzymes, such as cytochrome P450 (CYP).[2]

Benzbromarone (BBR) is used as a uricosuric agent due to its inhibition of the renal reabsorption of uric acid by the human uric acid transporter I (hURAT1). Thus, BBR is effective in the treatment of hyperuricemia and gout, particularly in cases in which allopurinol, a xanthine oxidase inhibitor, is ineffective. It has been used in Japan and in parts of Europe for a long period of time.[3] However, BBR is not approved for use in the United States, and it has been withdrawn from the market in Europe due to being responsible for fatal liver injury.[4,5] Although the specific mechanisms responsible for its hepatic toxicity are still unknown, mitochondrial toxicity and the formation of reactive metabolites have been hypothesized as potential causes of its toxicity.

In our previous study[6], we identified 2,6-dibromohydroquinone (DBH) and mono-debrominated catechol (CAT), formed via the *ipso*-substitution of BBR, as novel metabolites produced within rat and human liver microsomal systems. Considering that the cytotoxicity of CAT was found to be slightly less in HepG2 cells and that CAT was not produced by CYP2C9 recombinant enzymes, which are supposedly responsible for the toxicity of BBR, it was previously concluded that hepatocellular toxicity induced by BBR can be attributed to the formation of DBH or its oxidative metabolite, 2,6-dibromo-*p*-benzoquinone (DBBQ) (Scheme

1); however, other bioactivation pathways have been proposed by other research groups.[7-9]

The present study aimed to design and synthesize novel BBR analogues that are not subject to metabolic activation and, therefore, would not cause hepatotoxicity. In our previous study, we presented a possible mechanism of *ipso*-substitution by CYP responsible for metabolic reactions involving a variety of *ortho* or *para*-substituted phenols.[10-15] During the first step, one electron and one proton are abstracted from the phenol moiety, or a hydrogen radical is abstracted from a hydroxy group within a *para* or *ortho*-substituted phenol, by CYP to produce a phenoxy radical. This radical is delocalized on the aromatic ring and is distributed to the *ipso*-position, followed by the rebound of the hydroxyl-radical equivalent on the heme iron of CYP to produce a semiquinol intermediate, which degrades into hydroquinone (catechol) or benzoquinone and thereby results in the elimination of the substituent.[12] Similarly, in the case of BBR, a phenoxy radical on the dibromophenol ring is formed during the first step, followed by the distribution of the radical to the *ipso*-position of the carbonyl group (*para*-position of hydroxy group). Then, the hydroxyl-radical equivalent on the heme iron attacks the *ipso*-position to produce an α-hydroxyketone, which is easily degraded into DBH or DBBQ via *ipso*-substitution and results in the cleavage of the carbon-carbon bond (Scheme 1). It was clear that the presence of a hydroxy group at the *para*-position of the carbonyl group of BBR is necessary for the *ipso*-substitution reaction that leads to the metabolic activation of BBR into DBH or DBBQ. Therefore, we designed multiple BBR derivatives in which the hydroxy group was transferred to the *meta*-position of the carbonyl group (Fig. 1), which would prevent metabolic activation because the radical would not be distributed to the *ipso*-position of the carbonyl group and *ipso*-substitution could not occur at the root of the carbonyl group.



Scheme 1 Proposed mechanism for metabolic activation of BBR via *ipso*-substitution.

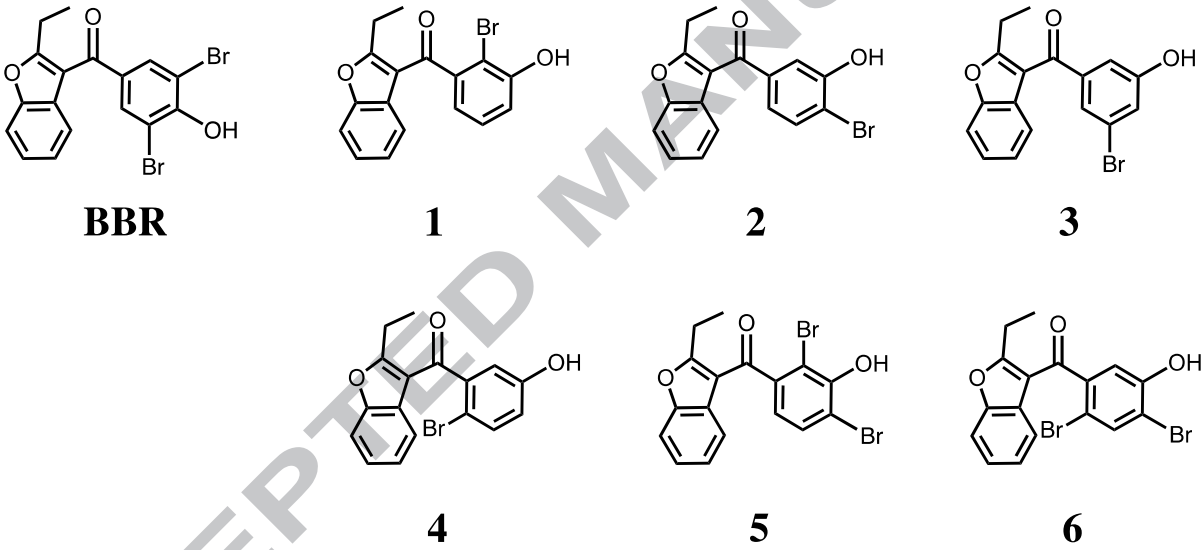


Fig. 1 Chemical structure of BBR derivatives.

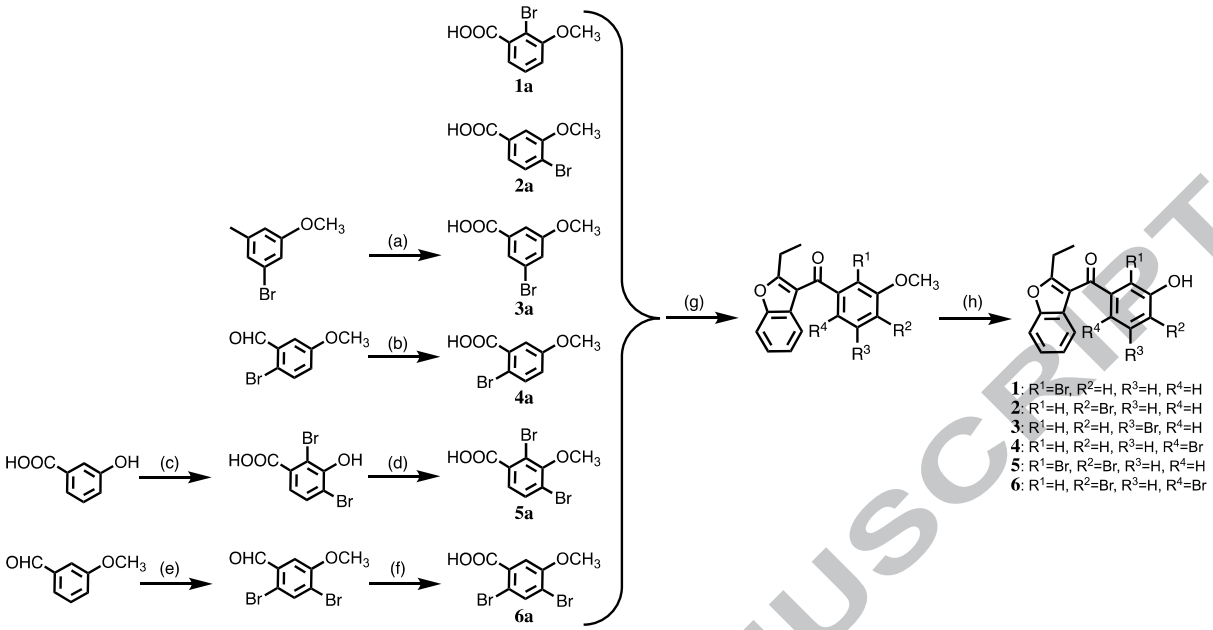
In the present study, we synthesized six novel BBR derivatives (**1-6**), designed to avoid metabolic activation via *ipso*-substitution, that were evaluated for their degree of toxicity and hURAT1 inhibition. The purpose of this study was to transform benzbromarone into compounds that have a reduced risk of hepatotoxicity while retaining efficacy.

Synthesis of BBR derivatives. We designed and chemically synthesized six BBR derivatives (**1-6)** that are shown in Fig. 1. The hydroxy group was moved to the *meta*-position of the carbonyl group in all of the compounds in order to avoid metabolic activation. The bromine atoms were retained within the molecules to minimize any changes in their physicochemical

properties, with the aim of producing levels of hURAT1 inhibition and pharmacokinetics that were similar to those of BBR. The bromine atom may be subject to *ipso*-substitution that could produce catechol metabolites; however, as described above, it is hypothesized that these metabolites would not contribute greatly to toxicity. Compounds **1**-**4** are structural isomers that have a bromine atom at different sites on the phenol ring, while compounds **5**-**6** contain two bromine atoms.

For the synthesis of the BBR derivatives, six different (di)bromomethoxybenzoic acids (**1a**-**6a**) were synthetically prepared as shown in Scheme 2, with the exception of 2-bromo-3-methoxybenzoic acid (**1a**) and 4-bromo-3-methoxybenzoic acid (**2a**), which were commercially available. With respect to compound **3**, 3-bromo-5-methoxybenzoic acid (**3a**) was prepared by the oxidation of 1-bromo-3-methoxy-5-methylbenzene with $KMnO_4$. In the case of compound **4**, 2-bromo-5-methoxybenzoic acid (**4a**) was prepared via the Pinnick oxidation of 2-bromo-5-methoxybenzaldehyde. For the synthesis of compound **5**, 3-hydroxybenzoic acid was used as the starting material. The methylation of 3-hydroxybenzoic acid after selective dibromination at the position *ortho* to the hydroxy group of 3-hydroxybenzoic acid with *N*-bromosuccinimide (NBS) produced 2,4-dibromo-3-methoxybenzoic acid (**5a**). Regarding compound **6**, bromination with NaBr and Oxone® followed by purification using silica gel column chromatography produced 2,4-dibromo-5-methoxybenzaldehyde, which was further oxidized via Pinnick oxidation to produce 2,4-dibromo-5-methoxybenzoic acid (**6a**).

After the treatment of these six benzoic acid derivatives (**1a**-**6a**) with $SOCl_2$, Friedel-Crafts acylation with 2-ethylbenzofuran was directed to the 3-position of the benzofuran ring. Subsequent demethylation of the methoxy groups with BBr_3 produced compounds **1**-**6**.



Scheme 2 Synthesis of BBR derivatives. (a) $KMnO_4$, pyridine/H_2O (2:5), reflux, 48 hr; (b) $NaClO_2$, NaH_2PO_4, *tert*-butyl alcohol, 2-methyl-2-butene, THF, H_2O, rt, 1 hr; (c) NBS, conc. H_2SO_4, 50 °C, 1 hr; (d) CH_3I, DMF, rt, 1 hr, then, NaOH, CH_3OH, H_2O, rt, 4 hr; (e) Oxone®, NaBr, CH_3OH/H_2O (1:1), rt, 1.5 hr; (f) $NaClO_2$, NaH_2PO_4, *tert*-butyl alcohol, 2-methyl-2-butene, THF, H_2O, rt, 45 min; (g) $SOCl_2$, reflux, 2-6 hr, then, 2-ethylbenzofuran, $SnCl_4$. CH_2Cl_2, rt, 2.5-24 hr; (h) BBr_3, CH_2Cl_2, rt, 1-3 hr. More details are described in Supplemental data.

Cytotoxicity of BBR derivatives in mouse hepatocytes. The cytotoxicity of BBR and compounds **1-6** at concentrations of 1 μM or 5 μM was assessed by measuring the lactate dehydrogenase (LDH) activity after 16 hr of drug exposure in hepatocytes that were prepared from mice treated with dexamethasone (DEX) to induce the production of CYP enzymes. BBR caused a dose-related LDH release of 27% and 52% at 1 μM and 5 μM, while compounds **1-6** showed less than 16% even at 5 μM. Although there was no concentration dependence in some compounds, it is clear that the cellular toxicities of compounds **1-6** are much lower than that of BBR, which indicates that **1-6** are much safer for metabolism by hepatocytes, as was expected (Fig. 2).

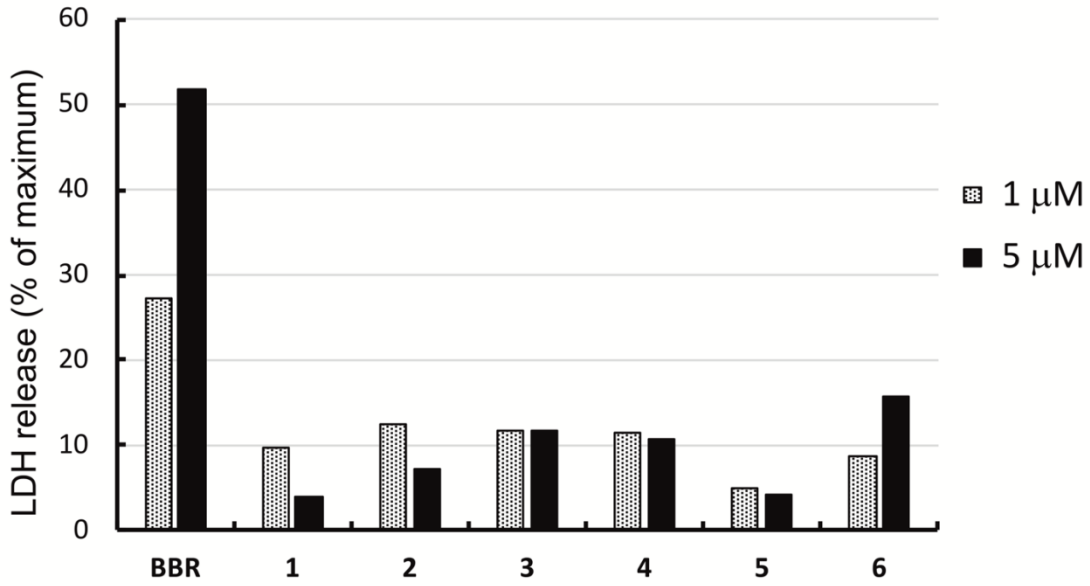


Fig. 2 Cytotoxicity of BBR derivatives in DEX-treated mouse hepatocytes. Cell toxicity was assessed by measuring LDH release after 18 hr of drug exposure. The data represent the average of measurements made in duplicate experiments.

Induction of NADPH-dependent mitochondrial permeability transition (MPT) by BBR derivatives in rat liver microsomes. Next, we evaluated the mitochondrial toxicity of the synthesized compounds compared to that of BBR; the hepatic toxicity of BBR has been hypothesized to be associated with its mitochondrial toxicity and metabolic activation.[16] The induction of MPT is one of the mechanisms that underlies drug-induced mitochondrial toxicity and is caused by many toxic drugs, including anticancer drugs, nonsteroidal anti-inflammatory drugs, and some thiazolidinones. The loss of mitochondrial membrane impermeability is associated with pathways involved in cell death, and MPT is thought to be a key event in this process.[16] In the present study, we investigated MPT induction after the incubation of liver microsomes with BBR derivatives according to a previously reported method[16] to evaluate the mitochondrial toxicity of the derivatives and their metabolites.

Initially, either BBR or compounds **1-6** (50 μM) were incubated with DEX-treated rat liver microsomes in the presence or absence of NADPH. After incubation for 30 min, the isolated mitochondrial fraction was added and the mitochondrial swelling was measured to determine the level of MPT induction. To evaluate the induction of MPT, we plotted the change in absorbance (540 nm) over time, then calculated the integral area over the curve (AOC). In the absence of NADPH, no significant differences were observed in the levels of MPT induced by any of the compounds, including BBR. On the other hand, the greatest level of NADPH-dependent, drug-induced MPT was observed for BBR, while no NADPH-dependent MPT induction was observed for compounds **1-6** (Fig. 3). These results suggest that compounds **1-6** are not subject to metabolic activation and have a lower potential to cause metabolite-induced mitochondrial toxicity than BBR.

As discussed above, the exact mechanisms underlying the hepatocellular toxicity of BBR are still unknown; multiple bioactivation pathways have been proposed by a number of research groups.[7-9] In our previous study, we demonstrated that metabolites formed via the *ipso*-substitution of BBR by CYP2C9 are cytotoxic (Scheme 1).[6] The fact that mitochondrial toxicity in hepatocytes treated with compounds **1-6**, which were designed to avoid *ipso*-substitution, was reduced clearly indicates that the metabolism of BBR via *ipso*-substitution is a major contributor to its hepatocellular toxicity.

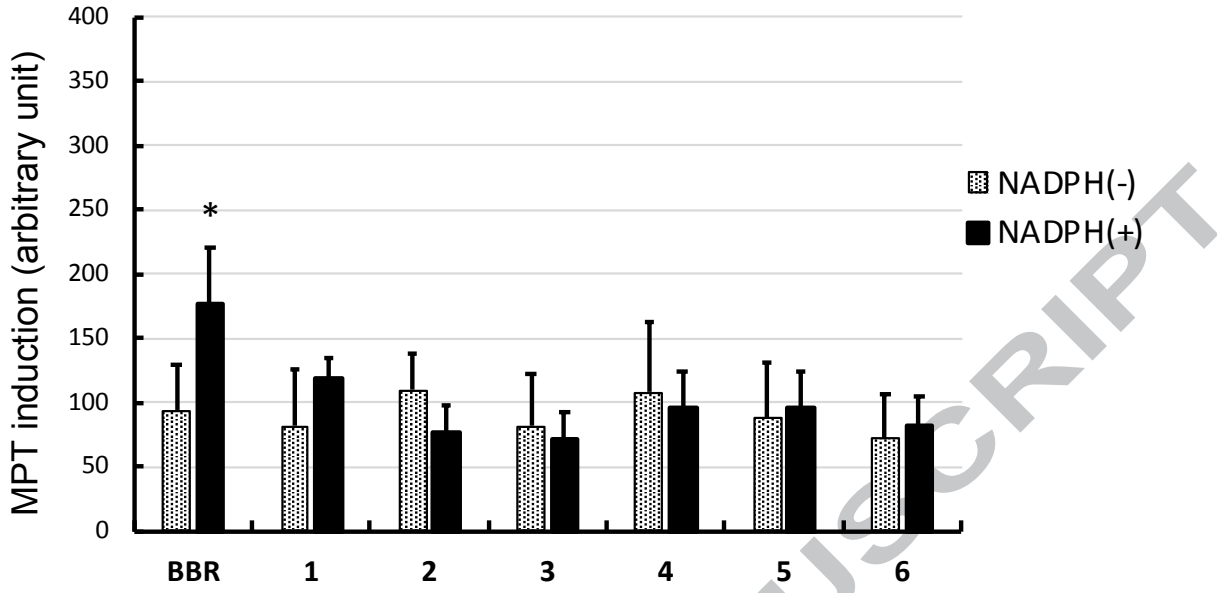


Fig. 3 NADPH-dependent MPT induction by BBR derivatives. The compounds (50 μM) were incubated with dexamethasone-treated rat liver microsomes at 37 °C in the absence or presence of NADPH for 30 min, and the isolated mitochondria were added at the indicated time point. Data represent the mean ± SD (n = 3). *: $p < 0.05$ vs. NADPH (-).

Inhibitory effects of the BBR derivatives on uric acid uptake by hURAT1-expressing HEK cells. To examine whether the inhibition of hURAT1 by BBR was retained in compounds **1-6,** we evaluated uric acid uptake in the presence of BBR or the synthesized compounds in HEK cells that overexpressed hURAT1 (10 μM), using a previously reported method.[17] As shown in Fig. 4, BBR strongly inhibited the uptake of uric acid to a level that was less than 10% of that of the control. While slightly weaker than BBR, compounds **1-6** also produced significant inhibition of uric acid uptake. There were no significant differences in the inhibitory potencies of the compounds.

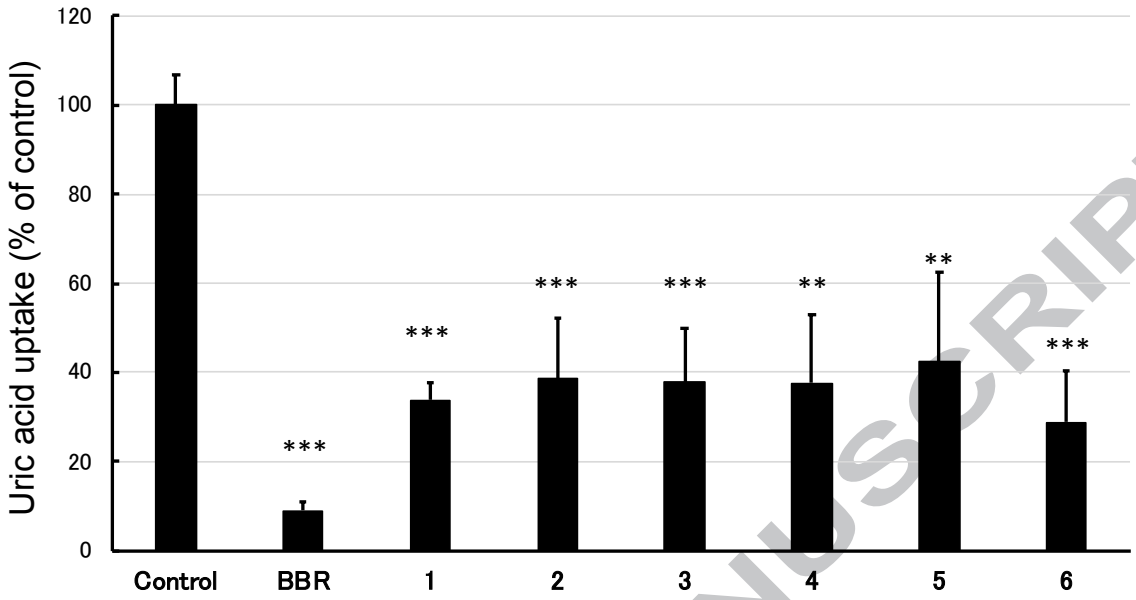


Fig. 4 Inhibitory effects of the BBR derivatives (10 μM) on uric acid uptake by hURAT1-expressing HEK cells. The uric acid uptake values were expressed as percentage relative to that obtained from the control (0.5% DMSO with no inhibitor). The data represent the mean ± SD (n = 4). **: $p < 0.01$ vs. control, ***: $p < 0.001$ vs. control.

In summary, we synthesized a novel set of BBR derivatives that were designed to avoid metabolic activation via *ipso*-substitution and found that they exhibited lower cytotoxicity in mouse hepatocytes; we also found that their metabolites exhibited lower mitochondrial toxicity than that of BBR. The BBR derivatives also showed significant hURAT1 inhibitory activity, while slightly weaker than BBR. Although the weaker pharmacological activities are unfavorable profiles for the alternative drugs aiming to mitigate the hepatotoxicity of BBR with retaining efficacy, we propose that these derivatives might serve as lead compounds that could be investigated for use as effective uricosuric agents with much better safety profiles than BBR, with the aim of enabling more rapid dose escalation *in vivo* and overcoming the attenuation of potency *in vitro*. The *in viv*o pharmacology and safety studies of the derivatives are currently underway.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/***

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Figure captions

Scheme 1 Proposed mechanism for metabolic activation of BBR via *ipso*-substitution.

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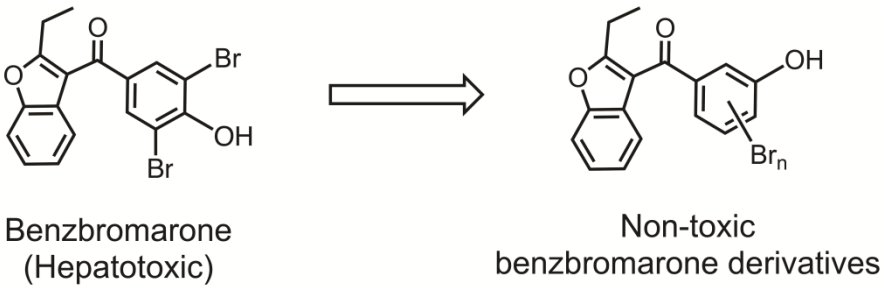

Benzbromarone (Hepatotoxic)

Non-toxic benzbromarone derivatives

- **Synthesis of a novel set of benzbromarone derivatives**
- **Reduced cytotoxicity in mouse hepatocytes**
- **Attenuated mitochondrial toxicity with metabolic activation**
- **Maintained activity of inhibition toward uric acid transporter**