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In vitro photobiochemical characterization of sulfobutylether-β-cyclodextrin formulation of bufexamac

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ABSTRACT

The present study aimed to modulate the photoreactivity of bufexamac, with a focus on photostability and phototoxicity, by forming an inclusion complex with sulfobutylether-β-cyclodextrin (SBECD). The photobiochemical properties of bufexamac were evaluated by reactive oxygen species (ROS) assay and using *in vitro* photogenotoxic assessment tools. To assess the inclusion properties of SBECD complex with bufexamac, a UV absorption spectroscopic study was also carried out. The influence of SBECD on the photoreactivity of bufexamac was analyzed by ROS assay and photostability test. From the photobiochemical data, superoxide generation from irradiated bufexamac indicated its photoreactivity; however, the photogenotoxic risk of bufexamac was negligible owing to low DNA-binding affinity and DNA-photocleaving activity. SBECD complex of bufexamac was formed, and the association constant of the complex was calculated to be 620 M^{-1}. On the basis of the photochemical data on bufexamac co-existing with SBECD, ROS generation from irradiated bufexamac (200 μM) was inhibited by SBECD at concentrations of over 20 μM. The degradation constant of bufexamac in SBECD was decreased ca. 30% compared with that of bufexamac, suggesting improvement of its photostability. The phototoxic risk of bufexamac might be attenuated by SBECD complexation, and cyclodextrin inclusion complexes might be a useful approach for modulating the phototoxicity of drugs.

1. Introduction

Photochemical reactions of pharmaceutics, including photodegradation and phototoxicity, are severe problems in terms of stability and safety in the pharmaceutical industry, and their possible cascade has been reported [1,2]. Drugs are excited by UVA (320–400 nm) and UVB (290–320 nm), and then the drugs directly/indirectly react with molecules, resulting in photodegradation and phototoxicity [1]. Reactive oxygen species (ROS) have been reported as one of the major causative intermediate species for photochemical reactions [3], and the ROS generation from irradiated chemicals induce the oxidation of various molecules. Notably, excited compounds react with biomolecules, leading to phototoxic skin responses, including photoirritation, photoallergy, and photogenotoxicity [1,4]. Several classes of pharmaceuticals, such as diuretic agents [5], non-steroidal anti-inflammatory drugs (NSAIDs) [6], and tricyclic antidepressants [7], exhibit some or all of the phototoxic reactions. Recently, for evaluating the photoreactivity of pharmaceutics, a ROS assay was proposed as a new photochemical assessment tool [1,8] to monitor ROS generation from irradiated compounds, including both singlet oxygen and superoxide. There appeared to be a good relationship between ROS generation and occurrences of phototoxic events for a number of known phototoxic compounds [8].

In previous studies, inclusion complexes of drugs with cyclodextrins (CyDs) were used for modulating the photoreactivity of pharmaceutics, such as naproxen, amlodipine, flutamide and curcuminoids [9–12]. Notably, phototoxic skin reactions of topically administered drugs are a critical hazard, and the adverse effects should be avoided. Thus, CyD complexations might modulate the phototoxic risk of topically administered compounds. Bufexamac, the model compound in the present study (Fig. 1), is administered topically on the skin in clinical use; however, phototoxic skin event of bufexamac has been reported [13]. The purpose of the present study was to control the phototoxic potential of bufexamac by using complexation with sulfobutylether-β-cyclodextrin (SBECD), a β-CyD derivative. The photochemical behavior of bufexamac was assessed by ROS assay. For assessment of photogenotoxic potential, the interaction of bufexamac with DNA was assessed by circular dichroism (CD) analysis and DNA-binding assay [14] and

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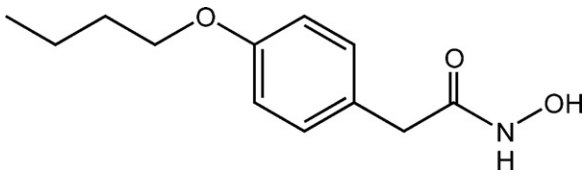

Fig. 1. Structure of bufexamac.

bufexamac-induced DNA photocleavage was evaluated by agarose gel electrophoresis. SBECD inclusion complex of bufexamac was formed and its inclusion properties were estimated using a spectroscopic study [15]. Possible changes of the photoreactivity of bufexamac with SBECD were evaluated by ROS assay, and the photostability of bufexamac with or without SBECD was monitored by ultra performance liquid chromatography equipped with electrospray ionization mass spectrometry (UPLC/ESI-MS).

2. Materials and methods

2.1. Chemicals

Bufexamac was purchased from Sigma (St. Louis, MO, USA). SBECD was supplied by Pfizer Inc. Salmon sperm DNA, plasmid pBR322 DNA, imidazole, p-nitrosodimethylaniline (RNO), nitroblue tetrazolium (NBT), Tween 20, disodium hydrogenphosphate 12-water, and sodium dihydrogenphosphate dihydrate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Ethidium bromide (EtBr) and agarose L03 were purchased from Nippon Gene (Toyama, Japan) and Takara Bio (Shiga, Japan), respectively. Acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). A quartz reaction container for high-throughput ROS assay was constructed by Ozawa Science (Aichi, Japan).

2.2. Determination of reactive oxygen species (ROS)

Singlet oxygen was determined following the Kraljic and ElMoshni procedure [16]. Briefly, samples containing bufexamac with or without SBECD, RNO (50 μM), and imidazole (50 μM) in 20 mM sodium phosphate buffer (NaPB, pH 7.4) were irradiated with UVA/B (30,000 lx) in a Light-Tron Xenon (LTX-01, Nagano Science, Osaka, Japan), and then UV absorption at 440 nm was measured using a SpectraMax plus 384 microplate spectrophotometer (Molecular Devices, Kobe, Japan).

Superoxide anion was also determined according to the Pathak and Joshi procedure [17]. Samples containing bufexamac (free or in SBECD inclusion complex) and NBT (50 μM) in 20 mM NaPB (pH 7.4) were irradiated with UVA/B (30,000 lx) for the indicated periods, and the reduction of NBT was measured by the increase of their absorbance at 560 nm, using SpectraMax plus 384 microplate spectrophotometer.

2.3. Circular dichroism (CD) analysis of DNA

Salmon sperm DNA with or without bufexamac was dissolved in 20 mM NaPB (pH 7.4), and CD spectra (average of ten scans) were collected from samples (2.4 mL) at 0.4 nm intervals between wavelengths of 200 and 350 nm using a Jasco model J-600 spectropolarimeter. Measurement was carried out at room temperature, and a baseline spectrum was subtracted from the collected data.

2.4. DNA-binding assay

The affinity of drugs for salmon sperm DNA was determined by the competitive binding study. For competitive binding experiments, 10 μL of DNA solution at a concentration of 100 μg/mL, dissolved in 20 mM NaPB (pH 7.4), was mixed with 20 μL of the tested drug at various final concentrations ranging from 0 to 2 μM in a 96-well microplate (AGC TECHNO GLASS, Chiba, Japan), then 70 μL of EtBr (7.0 μM) was added to the assay mixture. The mixture was incubated for 15 min at 37 °C. After incubation, the fluorescence (excitation, 550 nm, and emission, 590 nm) of each mixture (100 μL) in 96-well microplates was measured with a Multilabel Counter (PerkinElmer, Norwalk, CT, USA).

2.5. DNA-photocleavage assay

The sample containing pBR322 DNA (10 μg/mL) and bufexamac (200 μM) in Tris–acetic acid–EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) was irradiated with UVA/B (375 kJ/m^2) in an Atlas Suntest CPS+ solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon arc lamp (1500 W). After the irradiation test, irradiated plasmid pBR322 DNA was separated by electrophoresis (0.8% agarose gel in TAE buffer), visualized with EtBr staining, and analyzed with image analyzing software Image J.

2.6. Determination of stoichiometry and the association constant

Bufexamac (0.5 mM) was dissolved in 20 mM NaPB (pH 7.4) containing 5% acetonitrile with SBECD (5, 10, 15, 20, 25, and 30 mM). Solutions containing the same concentrations of SBECD without bufexamac were also prepared. UV–Vis absorption spectra were recorded with a HITACHI U-2010 spectrophotometer (HITACHI, Tokyo, Japan) interfaced to a PC for data processing (Software: Spectra Manager). Spectrofluorimeter quartz cell with 10 mm pathlength was employed. The spectra of bufexamac were obtained by the subtraction of the spectra of SBECD from those of complex for removal of the contribution of SBECD. The obtained UV absorption of bufexamac at 278 nm (A_{278}) was substituted into the following Scott's equation, and described on Scott's plot [15]:

$$[\text{SBECD}] \cdot [\text{Buf}] \cdot \frac{L}{A_{278}} = \frac{1}{\varepsilon}[\text{SBECD}] + \frac{1}{K \cdot \varepsilon}$$

where [SBECD] and [Buf] indicate the molar concentrations of SBECD and bufexamac (mM), respectively. L is the light path length, ε equals to the molar extinction coefficient, and K represents the association constant. Then, the values of K and stoichiometry were obtained from Y-intercept/slope and linearity of Scott's plot, respectively.

2.7. Photostability testing

For photostability testing, the solutions of bufexamac (1 mg/mL) and its SBECD inclusion complex (equimolar ratio between bufexamac and SBECD) were dissolved in water containing 50% acetonitrile in a 1.5 mL clear glass vial (12 mm × 32 mm, Shimadzu, Kyoto, Japan). The samples were stored in the Atlas Suntest CPS+ solar simulator, and photostability testing was carried out at 25 °C with an irradiance of 750 W/m^2 for the indicated times (0, 15, 30, 60, and 120 min). The irradiated and non-irradiated samples were subjected to UPLC analyses to determine the amounts of remaining bufexamac. All analyses were performed on a Waters Aquity UPLC™ system (Waters, Milford, MA), which includes a binary solvent manager, a sample manager, a column compartment, and a Micromass SQ detector connected with a Waters Masslynx v 4.1. A Waters Acquity UPLC™ BEH C_{18} (particle size: 1.7 μm, column size: ϕ2.1 mm × 50 mm; Waters) was used, and the column temperature was maintained at 40 °C. The standards and samples were separated using a gradient mobile phase consisting of Milli-Q containing 0.1% formic acid (A) and methanol (B). The gradient condition of

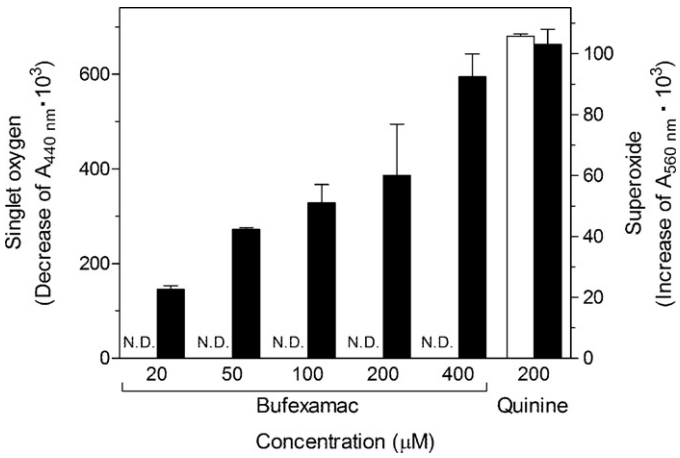

Fig. 2. Generation of ROS from photoirradiated bufexamac and quinine. Each chemical was dissolved in 20 mM NaPB (pH 7.4) at the indicated concentrations, and then exposed to simulated sunlight (30,000 lx). Open bar, singlet oxygen; and filled bar, superoxide. Data represent mean ± S.D. of three experiments.

the mobile phase was 0–0.5 min, 50% A; 0.5–3.5 min, 50–5% A; 3.5–5 min, 5%, and the flow rate was set at 0.25 mL/min.

2.8. Data analysis

For statistical comparisons, a one-way analysis of variance (ANOVA) with the pairwise comparison by Fisher's least significant difference procedure was used. A *P* value of less than 0.05 was considered significant for all analyses.

3. Results

3.1. Photochemical reactions of bufexamac

The ROS assay enabled to identification of the type of photochemical reaction by monitoring the generation of singlet oxygen through type II photochemical reaction and superoxide through type I photochemical reaction. In the present study, the generation of ROS from bufexamac was detected by ROS assay to clarify the type of photochemical reaction for bufexamac (Fig. 2). Exposure of quinine, a known phototoxic drug, to simulated sunlight resulted in the generation of both singlet oxygen and superoxide; however, bufexamac could generate only superoxide in a concentration-dependent manner. The results suggested that bufexamac would mainly induce type I photochemical reaction. The ROS-generating behavior of bufexamac ($^1O_2$, $\Delta_{440} \times 10^3$: not detected, O_2^-, $\Delta_{560} \times 10^3$: 60) was similar to that of carbamazepine ($^1O_2$, $\Delta_{440} \times 10^3$: not detected, O_2^-, $\Delta_{560} \times 10^3$: 96), a phototoxic drug [18], at a concentration of 200 μM [19]. Thus, bufexamac was found to be photoreactive and/or phototoxic, and the result was in agreement with a previous clinical report [13].

3.2. Photogenotoxic potential of bufexamac

For further photochemical characterization, the interaction of bufexamac with DNA was evaluated by DNA-binding assay [14], and nalidixic acid, which has the affinity to DNA, was used as a positive control (Fig. 3A). The emission of intense fluorescence from ethidium (4.9 μM) was observed in the presence of DNA (10 μg/mL). The addition of nalidixic acid induced a decrease of fluorescence in a concentration-dependent manner; however, no significant changes of fluorescence emission were observed for bufexamac, suggesting low affinity of bufexamac to DNA. To clarify the interaction of bufexamac and DNA, CD spectral analysis on DNA

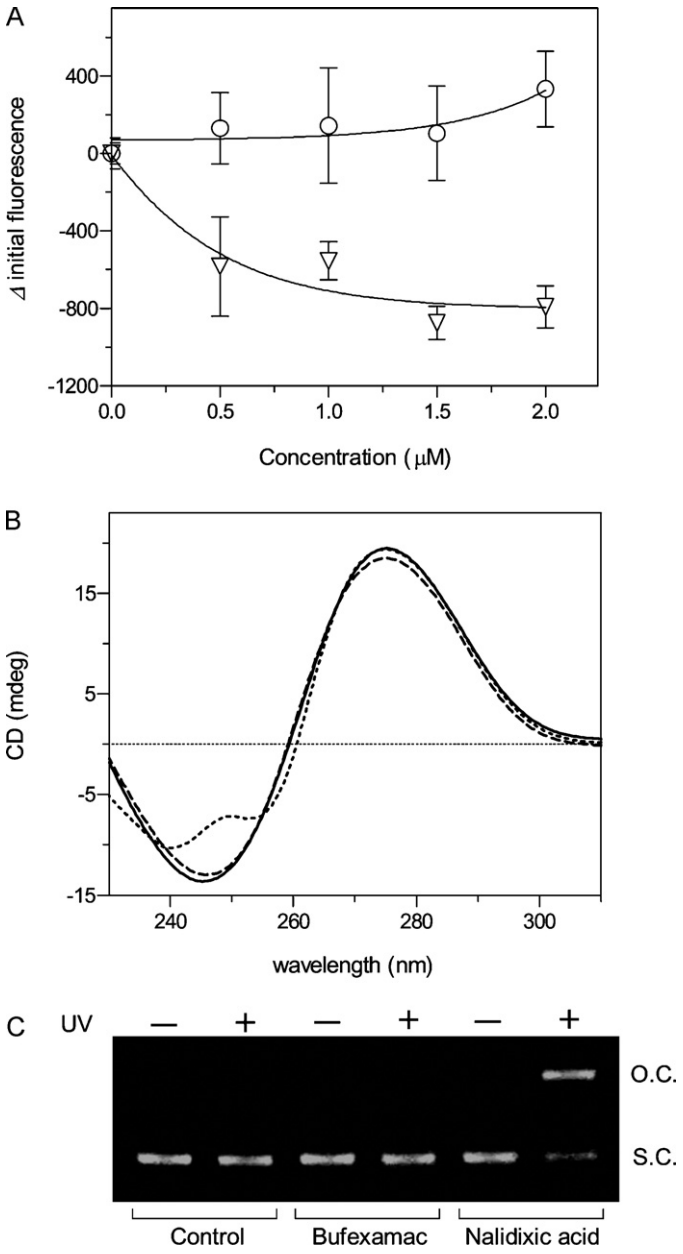

Fig. 3. *In vitro* photogenotoxic assessments. (A) Typical ethidium displacement curves for chemicals. The binding of ethidium (4.9 μM) to DNA was inhibited by increasing concentrations of nalidixic acid, but not by bufexamac. (○) Bufexamac and (▽) nalidixic acid. Data represent mean ± S.D. of four experiments. (B) CD spectra representative of DNA (100 μg/mL) in the presence of compounds (100 μM). Solid line, DNA alone; dashed line, DNA in the presence of bufexamac; and dotted line, DNA in the presence of nalidixic acid. (C) Photodynamic impairment of plasmid pBR322 DNA induced by irradiated compounds. Supercoiled DNA was exposed to UV with/without compounds. EtBr-stained 0.8% agarose gels are shown. O.C., open circular form; and S.C., supercoiled form.

(100 μg/mL) with or without compounds (100 μM) was also carried out (Fig. 3B). A solution of DNA exhibits a positive band at 275 nm due to base stacking and a negative band at 248 nm due to the helicity, which is characteristic of DNA in the right-handed B form [20]. Adding nalidixic acid to DNA solution, the intensity of the negative band at 248 nm decreased, suggesting the structural changes of DNA. In contrast, no spectral transitions were observed for bufexamac, suggesting weak interaction of bufexamac with DNA.

To validate the photogenotoxic risk of bufexamac, the conversion of plasmid pBR322 DNA from supercoiled (SC) form to open circular (OC) form was also analyzed by AGE (Fig. 3C). DNA damage

was clearly induced by nalidixic acid after exposure to simulated sunlight, and the conversion of pBR322 DNA from the SC to the OC form was estimated to be ca. 64% on the basis of the band intensity. In contrast, bufexamac-induced DNA photocleavage was not observed, suggesting that bufexamac is less photogenotoxic. Overall, bufexamac exhibited neither interaction with DNA nor DNA-photocleaving activity, and bufexamac may not cause photogenotoxicity.

3.3. Stoichiometric analysis of bufexamac–SBECD inclusion complex

The photobiochemical data of bufexamac indicated photodegradative and phototoxic potentials, except photogenotoxic risk, and SBECD complexation was applied for modulating the photoreactivity of bufexamac in this study. Generally, there is an optimal molar ratio between CyD and chemicals for forming an inclusion complex; therefore, a spectroscopic method using the changes of UV-absorption spectra and Scott's plot was used for evaluating the stoichiometry of the inclusion complex in the present investigation [15]. The UV spectral patterns of SBECD–bufexamac complex were recorded in 20 mM NaPB (pH 7.4) (Fig. 4A). On the basis of UV spectral data, hyperchromicity and slight bathochromicity were observed ranging from 250 nm to 300 nm, and strong absorption was detected at approximately 278 nm; these effects suggested that the UV absorbability of bufexamac was changed by SBECD. To obtain the stoichiometry and association constant of the SBECD inclusion complex of bufexamac, Scott's plot was described using the UV absorption data and the concentrations of bufexamac and SBECD (Fig. 4B). The plot of SBECD concentration versus [SBECD]·[Buf]/Absorbance exhibited linearity, and its correlation coefficient was estimated to be 1.00. Generally, the inclusion ratio between CyD and compound is stoichiometrically determined to be 1:1 when Scott's plot is indicative of the linearity [21]. In addition to the stoichiometry of the inclusion complex, the value of K of the inclusion complex was also calculated to be 620 M^{-1} from the present analysis. On the basis of the data obtained, SBECD forms a 1:1 inclusion complex with bufexamac, and the complex should be relatively stable.

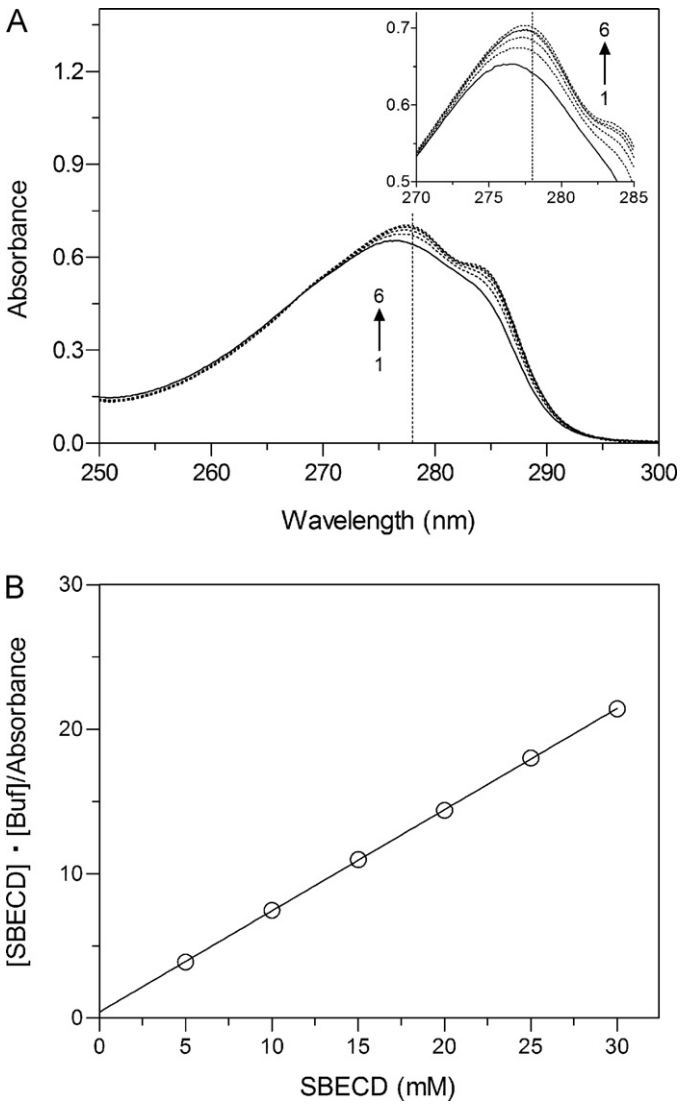

Fig. 4. Stoichiometry analysis of SBECD inclusion property for bufexamac. (A) UV spectral patterns of bufexamac (0.5 mM) with SBECD. Concentration of SBECD: (1) 5, (2) 10, (3) 15, (4) 20, (5) 25, and (6) 30 mM. (1) Solid line; (2)–(6) dotted line. (B) Scott's plot for interaction between bufexamac and SBECD by UV spectrometry.

3.4. Inhibitory effect of SBECD on ROS generation from bufexamac

Although SBECD complex with bufexamac could be formed, the influence of SBECD on the photochemical behavior of bufexamac is still unclear. Therefore, ROS generation from irradiated bufexamac (200 μM) co-existing with SBECD (ranging from 0 to 800 μM) was examined by ROS assay to clarify the possible transition of photoreactivity for bufexamac (Fig. 5). SBECD complexation led to suppression of superoxide generation from irradiated bufexamac in an SBECD-concentration-dependent manner. In detail, SBECD at concentrations of 20 and 100 μM exhibited significant reduction of superoxide generation from irradiated bufexamac by ca. 75 and 92%, respectively, and the generation of superoxide was negligible in the presence of SBECD at concentration of over 200 μM. On the basis of the data obtained, SBECD modulated the photoreactivity of bufexamac by forming an inclusion complex, and SBECD may attenuate bufexamac-induced phototoxic skin reactions by forming an inclusion complex when the complex is topically administered.

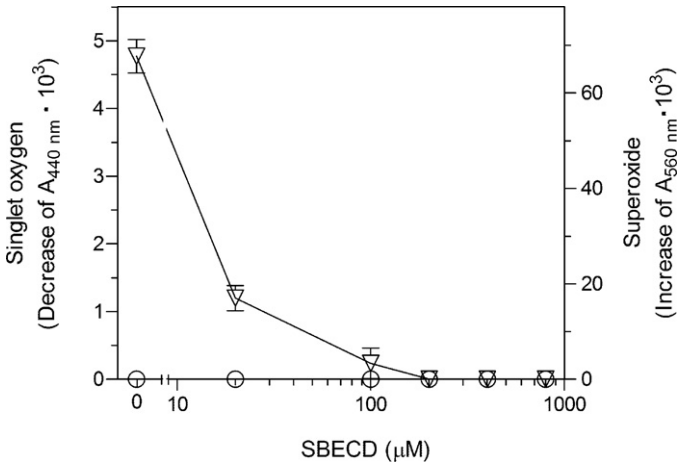

Fig. 5. Generation of ROS from irradiated inclusion complex of bufexamac with SBECD. SBECD was dissolved in 20 mM NaPB (pH 7.4) at the indicated concentrations with bufexamac (200 μM), and then exposed to simulated sunlight (30,000 lx). (○) singlet oxygen; and (▽) superoxide. Data represent mean ± S.D. of three experiments.

3.5. Photostability testing on bufexamac and its SBECD-inclusion complex

According to the ROS data, photoreactive and/or phototoxic potential of bufexamac is modulated by SBECD; this finding prompted us to clarify the photostability of bufexamac in SBECD. Solution-state photostability test using a solar simulator was

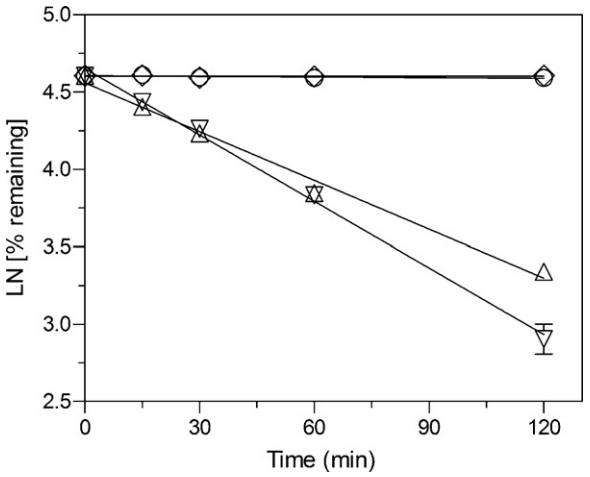

Fig. 6. Photodegradation profiles of bufexamac. Each sample was exposed to UVA/B (750 W/m^2) for the indicated periods, and the remaining bufexamac was evaluated by UPLC/ESI-MS. Bufexamac: (○) non-irradiated, and (▽) irradiated, and bufexamac–SBECD inclusion complex: (◊) non-irradiated, and (△) irradiated. Data represent mean ± S.D. of three experiments.

carried out on bufexamac with or without SBECD (Fig. 6). Degradation kinetics was calculated according to the following equation: $\ln A = \ln A_0 - kt$, where A is the remaining peak area of bufexamac, t is the time (min), and k is the slope (degradation constant). Both bufexamac solutions were stable without UV irradiation since the results of the remaining bufexamac were estimated to be almost 100% of initial bufexamac until 120 min in both bufexamac solutions. In contrast, solution-state bufexamac was rapidly photodegraded by exposure to UV, and the remaining bufexamac at 120 min was estimated to be ca. 18.3%. The solution-state bufexamac in SBECD was also photodegraded after UV irradiation, and the remaining bufexamac at 120 min was calculated to be ca. 28.2%. The degradation constants of bufexamac with and without SBECD were estimated to be 1.05×10^{-2} min^{-1} and 1.43×10^{-2} min^{-1}, respectively. There was ca. 30% reduction of the degradation constant of bufexamac by forming SBECD complexation; therefore, the photostability of bufexamac should be slightly improved by SBECD.

4. Discussion

In the present study, we first demonstrated that SBECD complexation was effective for controlling the phototoxicity of bufexamac. On the basis of the photobiochemical data, bufexamac generated superoxide; however, the photogenotoxic potential of bufexamac was not identified. SBECD inclusion complex of bufexamac was prepared, and the inclusion ratio was estimated to be 1:1 by a spectroscopic method. From the photochemical data on the inclusion complex, the photoreactivity of bufexamac might be modulated by equimolar SBECD.

Generally, CyDs are often applied for improving solubility, dissolution rate, bioavailability, and chemical stability, including hydrolysis oxidation and photodegradation of drugs [22,23]. In particular, β-CyD is considered a useful solubilizing agent because of its inclusion abilities; however, natural β-CyD has limited aqueous solubility due to relatively strong binding of the CyD molecules. To overcome this drawback, SBECD, an anionic β-CyD derivative, was synthesized for better solubilization than natural β-CyD, and it has been applied as a solubilizing agent for some pharmaceutics, such as ziprasidone, aripiprazole, and voriconazole [24].

On the basis of the ROS data, bufexamac was found to have photoreactivity, possibly leading to photodegradation and phototoxicity; however, the potent photogenotoxic risk of bufexamac was not observed in all the *in vitro* photogenotoxic assessment tools. Although the photogenotoxic risk of bufexamac was negligible, bufexamac should be indicative of photoreactivity mainly via type I photochemical reaction since bufexamac is topically used in clinical settings, and the phototoxic risk of bufexamac is recognized as one of its severe side effects. To attenuate the phototoxic potential of bufexamac, SBECD-based formulation was designed in the present investigation. On the basis of the physicochemical data, SBECD should form equimolar and stable inclusion complex with bufexamac. Photochemical properties of the inclusion complex were examined to clarify whether SBECD truly attenuated the phototoxicity of bufexamac. On the basis of the ROS data, generation of superoxide from bufexamac was completely inhibited by more than an equimolar concentration of SBECD. The photostability of bufexamac was also improved slightly by forming inclusion complex with SBECD owing to the ca. 30% reduction of the degradation constant of bufexamac. The results suggest that SBECD attenuated photoactivation of bufexamac and/or blocked interaction of excited bufexamac with oxygen owing to complexation with bufexamac. Overall, bufexamac in SBECD was found to be less photoreactive than bufexamac itself, and SBECD-based complexation might be effective for modulating the phototoxicity of bufexamac in terms of photosafety.

Topical application of chemicals on the skin provokes the concern about the occurrence of the phototoxic risk of the compounds because of direct exposure of the skin to both compounds and sunlight. Previously, Moore et al. reported that topically applied agents, such as ketoprofen, coumarin, and hydrocortisone, induced direct cutaneous phototoxicity [25]. According to the European Medicines Agency (EMEA) and the Food and Drug Administration (FDA) guidelines [26–28], topical application of compounds has been explicitly described as one of the conditions for testing chemicals; therefore, attenuation of the phototoxic risk of topically applied chemicals is required in terms of photosafety. Sunscreens are usually used to avoid drug-induced photodermatoses [25], and the present investigation suggested that CyD complexation may also be an effective approach for controlling the phototoxicity of topically administered drugs.

In conclusion, SBECD could inhibit ROS generation from irradiated bufexamac and slightly improve the photostability of bufexamac; therefore, the phototoxic risk of bufexamac could be reduced by SBECD complexation, and the SBECD-based formulation strategy might be effective for modulating the phototoxicity of bufexamac.

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