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Development of fluorometric reactive oxygen species assay for photosafety evaluation

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ABSTRACT

The present investigation involved an attempt to develop a new reactive oxygen species (ROS) assay system for the photosafety assessment of chemicals using 1,3-diphenylisobenzofuran (DPBF), a fluorescent probe for monitoring ROS generation. The assay conditions of the fluorometric ROS (fROS) assay were optimized focusing on the solvent system, concentration of DPBF, fluorescent determination, screening run time and reproducibility. The photoreactivity of 21 phototoxic and 11 non-phototoxic compounds was assessed by fROS assay, and the obtained ROS data were compared with the results from a micellar ROS (mROS) assay and *in vitro*/*in vivo* phototoxicity information to confirm the predictive capacity of the fROS assay. In the optimized fROS assay, intra-day and inter-day precision levels (coefficient of variation) were found to be below 5%, and the Z'-factor for DPBF fluorescence quenching showed a large separation between positive and negative controls. Of all tested compounds, 3 false positive and 7 false negative predictions were observed in the fROS assay, and the negative predictivity for the fROS assay was found to be lower than that for the mROS assay. Although the fROS assay has some limitations, the procedures for it were highly simplified with a marked reduction in screening run time and one analytical sample for monitoring ROS generation from compounds. The fROS assay has the potential to become a new tool for photosafety assessment at an early stage of product development.

1. Introduction

Several chemical products, including pharmaceutics and cosmetics, can induce phototoxic reactions in the skin and eyes after exposure to sunlight, consisting of partial ultraviolet (UV) B (290–320 nm), UVA (320–400 nm) and visible light (400–700 nm) (Epstein, 1983; Moore, 2002; Onoue et al., 2009). For photosafety evaluations, a UV absorption system (Henry et al., 2009) and a 3T3 neutral red uptake phototoxicity test (Spielmann et al., 1994) were recommended in the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2004). In addition to these recommended methods, interest in *in vitro* photosafety evaluations on the basis of the photochemical and photobiological mechanisms, notably the generation of reactive oxygen species (ROS) from photoirradiated chemicals, has increased in the pharmaceutical and cosmetic industries. A ROS assay was developed as an *in vitro* photoreactivity assessment tool for monitoring ROS generation from photoirradiated pharmaceuticals, including singlet oxygen and superoxide (Onoue and Tsuda, 2006), and the International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has recommended the ROS assay as a photosafety assessment tool in the ICH S10 guidelines for photosafety evaluation (ICH, 2013). The experimental conditions of the ROS assay were optimized (Onoue et al., 2008a; Onoue et al., 2008b) and validated (Onoue et al., 2013; Onoue et al., 2014a), offering high assay productivity and prediction capacity; however, the solubility issues of the ROS assay appeared in multi-laboratory validation studies (Onoue et al., 2013; Onoue et al., 2014a). To overcome these limitations, an albuminous ROS assay (Onoue et al., 2014b) and a micellar ROS (mROS) assay (Seto et al., 2013) were also developed for evaluation of the photoreactivity of poorly water-soluble chemicals, and it has been proposed that these ROS assay systems could have a wide range of applicability for photosafety assessment.

Although these ROS assay systems could be useful as early screening tools for photosafety assessment, challenges with the current ROS assay systems for screening purposes might still remain. In the current ROS assay systems, the preparation of two independent analytical samples is needed for monitoring the generation of both singlet oxygen and superoxide from photoirradiated chemicals, and high-energy UV irradiation is required for photosafety assessments of tested chemicals, leading to operational complexity and a long run time. To improve

Abbreviations: CV, coefficient of variation; DBB, *o*-dibenzoylbenzene; DMSO, dimethyl sulfoxide; DPBF, 1,3-diphenylisobenzofuran; fROS assay, fluorometric reactive oxygen species assay; H_2O_2, hydrogen peroxide; ICH, the International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; mROS assay, micellar reactive oxygen species assay; NaPB, sodium phosphate buffer; NBT, nitroblue tetrazolium; OECD, the Organisation for Economic Co-operation and Development; PABA, *p*-aminobenzoic acid; ROS, reactive oxygen species; UV, ultraviolet.

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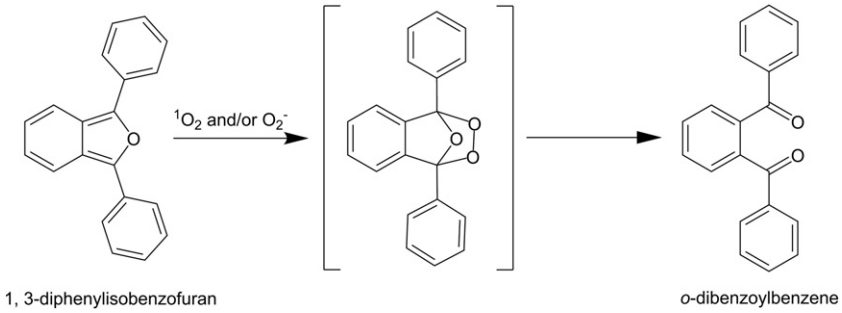

Fig. 1. Chemical structure and fluorescent quenching scheme of 1,3-diphenylisobenzofuran (DPBF).

these drawbacks, a novel screening strategy for monitoring ROS generation from photoirradiated chemicals would need to be developed. In the previous report, 1,3-diphenylisobenzofuran (DPBF; Fig. 1) had been used for an *in vitro* phototoxicity test on porous silicon nanoparticles (Xiao et al., 2011), and the decrease of absorbance at 410 nm caused by photobleaching of DPBF was monitored for detecting singlet oxygen generation from porous silicon nanoparticles. The operation time of the previous method was 10 min; thus the use of DPBF for detecting ROS generation may provide shorter operation time compared with the current ROS assay system. However, spectral interference between DPBF and test chemicals was concerned, possibly leading to false predictions; therefore, the colorimetrical methodology might not be appropriate for establishing a new photosafety assay. Recently, many types of fluorescent probe have been reported for detecting ROS in biological and non-biological samples (Gomes et al., 2005), and DPBF has been reported as a fluorescent probe for detecting ROS generation. DPBF in particular can detect both singlet oxygen (Wozniak et al., 1991) and superoxide (Ohyashiki et al., 1999) in phospholipid liposomes by its fluorescence decrease. DPBF changes to *o*-dibenzoylbenzene (DBB), a non-fluorescent substance, by reaction with singlet oxygen and/or superoxide. In general, fluorometric methods have higher detection sensitivity and specificity than colorimetric methods, and spectral interference can be avoided with the use of fluorometric methods. According to the previous reports, DPBF would be useful for monitoring both ROS generation from photoirradiated chemicals, and the use of a fluorescent probe might be of help to increase the productivity and usability of the ROS assay for photosafety assessments of chemicals.

The present study proposes a novel ROS assay system using DPBF, named fluorometric ROS (fROS) assay, for photosafety assessments of chemicals as an alternative to the current ROS assay systems. The assay conditions of the fROS assay were optimized, focusing on the solvent system, DPBF concentration, wavelength for the detection of DPBF fluorescence, irradiation time, sensitivity and robustness, and validation of this assay was also carried out. The fROS assay was applied to 21 phototoxic and 11 non-phototoxic compounds. To clarify the predictivity of the fROS assay, the photoreactivity of the tested chemicals was compared with the ROS data obtained from the mROS assay and *in vitro*/*in vivo* photosafety information.

2. Materials and methods

2.1. Chemicals

Chlorpromazine HCl, fenofibrate, hydrochlorothiazide, indomethacin, ketoprofen, lomefloxacin HCl, lovastatin, 6-methylcoumarin, omeprazole, pravastatin Na, cinnamic acid, erythromycin, histidine, *p*-aminobenzoic acid (PABA), penicillin G, phenytoin, dimethyl sulfoxide (DMSO), imidazole, nitroblue tetrazolium (NBT), *p*-nitrosodimethylaniline, Tween 20, disodium hydrogen phosphate 12-water and sodium dihydrogen phosphate dihydrate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cilnidipine, naproxen, benzocaine and sulisobenzone were purchased from Tokyo Chemical Industry (Tokyo, Japan). Diclofenac Na, doxycycline HCl, fluphenazine 2HCl, nalidixic acid, quinine HCl, sparfloxacin, aspirin, bumetrizole and chlorhexidine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Amlodipine, atorvastatin and enoxacin were purchased from LKT Laboratories (St. Paul, MN, USA). DPBF was obtained from Kanto Chemical (Tokyo, Japan).

2.2. Irradiation conditions

Chemicals were stored in an Atlas Suntest CPS+ solar simulator (Atlas Material Technology LLC, Chicago, IL, USA) equipped with a xenon arc lamp (1500 W) and cooling unit SR-P20FLE (Hitachi, Tokyo, Japan). A UV special filter (# 56052371, Atlas) was installed to adapt the spectrum of the artificial light source to natural daylight, and the Atlas Suntest CPS series has high irradiance capability that meets CIE85/1989 daylight simulation requirements. The irradiation test was carried out at 25 °C with irradiance of *ca.* 2.0 mW/cm^2 as determined using the calibrated UVA detector Dr. Hönle # 0037 (Dr. Hönle, Munich, Germany).

2.3. Fluorescence spectrum analysis

DPBF (10 μM) was dissolved in 20 mM sodium phosphate buffer (NaPB; pH 7.4) containing 0.5% (*v*/*v*) Tween 20 with or without 1.5% (*w*/*v*) hydrogen peroxide (H_2O_2), and the mixture was incubated at room temperature for 15, 30, 45, 60, and 90 min. These procedures were carried out in the dark. After incubation, fluorescence spectra of DPBF (excitation: 414 nm) were collected using SAFIRE (TECAN, Männedorf, Switzerland).

2.4. Fluorometric reactive oxygen species (fROS) assay

ROS generation from irradiated compounds was qualitatively monitored by conversion from DPBF to DBB. Each tested compound was dissolved in DMSO at 10 mM as a stock solution. Samples containing compounds (200 μM) and DPBF (10 μM) in 20 mM NaPB (pH 7.4) with 0.5% (*v*/*v*) Tween 20 were prepared in the dark. As a vehicle, a sample containing DPBF (10 μM) in 20 mM NaPB (pH 7.4) with 0.5% (*v*/*v*) Tween 20 was also prepared in the dark. The samples were irradiated with simulated sunlight for 1.5 min. Fluorescence from DPBF (excitation: 414 nm and emission: 487 nm) was measured using SAFIRE before and after irradiation. To monitor ROS generation from irradiated chemicals, the obtained data were analyzed using the following equations: (i) [Fluorescence data (% of initial)] = A/B × 100. A and B represent fluorescence values for samples before and after irradiation, respectively; (ii) [Fluorescence quenching (% of vehicle)] = (C–D)/C × 100. C and D represent fluorescence data (% of initial) for vehicle and tested chemical groups, respectively.