Toxicology in Vitro 27 (2013) 1838–1846

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Development of micellar reactive oxygen species assay for photosafety evaluation of poorly water-soluble chemicals

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ARTICLE INFO

Article history:
Received 19 February 2013
Accepted 16 May 2013
Available online 30 May 2013

Keywords:
Micellar solution
Photoreactivity
Photosafety assessment
Phototoxicity
Reactive oxygen species
Poorly water-soluble chemicals

ABSTRACT

A reactive oxygen species (ROS) assay was previously developed for photosafety assessment; however, the phototoxic potential of some chemicals cannot be evaluated because of their limited aqueous solubility. The present study was undertaken to develop a new micellar ROS (mROS) assay system for poorly water-soluble chemicals using a micellar solution of 0.5% (v/v) Tween 20 for solubility enhancement. In repeated mROS assay, intra- and inter-day precisions (coefficient of variation) were found to be below 11%, and the *Z′*-factors for singlet oxygen and superoxide suggested a large separation band between positive and negative standards. The ROS and mROS assays were applied to 65 phototoxins and 18 non-phototoxic compounds for comparative purposes. Of all 83 chemicals, 25 were unevaluable in the ROS assay due to poor solubility, but only 2 were in the mROS assay. Upon mROS assay on these model chemicals, the individual specificity was 76.5%, and the positive and negative predictivities were found to be 93.9% and 86.7%, respectively. The mROS assay provided 2 false negative predictions, although negative predictivity for the ROS assay was found to be 100%. Considering the pros and cons of these assays, strategic combined use of the ROS and mROS assays might be efficacious for reliable photosafety assessment with high applicability and predictivity.

1. Introduction

Several classes of pharmaceutics, cosmetics and food ingredients can be excited by sunlight, consisting of partial ultraviolet (UV) B (290–320 nm), UVA (320–400 nm) and visible light (400–700 nm); then, these photo-excited agents can elicit phototoxic reactions in skin and eyes (Epstein, 1983; Moore, 2002; Onoue et al., 2009). For photosafety evaluation, a number of effective *in vitro* methodologies have been proposed within the past few decades (Seto et al., 2012), and, notably, 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) guideline (OECD, 2004). Considering the implementation of the 3Rs principle (replacement, reduction and refinement), interest in the development of *in vitro* assessments based on photochemical and photobiological mechanisms should be increasing in photosafety assessments. A reactive oxygen species (ROS) assay was designed for the *in vitro* photoreactivity assessment of pharmaceuticals on the basis of ROS generation from photoirradiated chemicals, including singlet oxygen and superoxide (Onoue and Tsuda, 2006). The experimental conditions of the ROS assay were optimized (Onoue et al., 2008a,b) and validated (Onoue et al., in press), offering high assay productivity and prediction capacity.

Although the ROS assay demonstrated high prediction capacity for photosafety assessment, there appeared to be at least two assay limitations in a multi-laboratory validation study: (i) false positive predictions and (ii) solubility issues (Onoue et al., in press). Since the ROS assay is carried out in early phases of photosafety assessments, false positives would be re-evaluated by appropriate follow-up assessments. In this context, the former assay limitation might not be a severe problem. In contrast, the solubility issues would be a serious problem for reliable photosafety assessment. In the validation study (Onoue et al., in press), 43% of tested chemicals could not be dissolved in reaction mixtures at 200 μM owing to their poor water solubility, and, additional experiments on these chemicals had to be performed at lower concentrations (20 or 2 μM). The ROS data on some phototoxins at lower concentrations led to different observations among three laboratories, and ROS

Abbreviations: CV, coefficient of variation; DMSO, dimethyl sulfoxide; 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; SD, standard deviation; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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http://dx.doi.org/10.1016/j.tiv.2013.05.008

data from chemicals at lower concentrations might not be suitable for photosafety assessment. Hence, appropriate modifications to the ROS assay system for enhanced applicability would be required for reliable photosafety assessment on poorly water-soluble chemicals.

For solubilizing poorly water-soluble drugs in oral and injectable solution forms, micelle systems are widely used in commercially available formulations (Strickley, 2004). In addition, a previous study demonstrated that the use of micellar solution systems, such as Tween 20, sodium laurate and sodium dodecyl sulfate (SDS), would be effective for monitoring singlet oxygen generation from poorly water-soluble chemicals because of the intense solubilizing potency and production of the biomembrane-mimetic environment (Onoue et al., 2008c). Thus, the present study attempted to develop a micellar ROS (mROS) assay with the aim of overcoming solubility issues of ROS assay, and thus a micellar solution of Tween 20 (polyoxyethylene sorbitan monolaurate), a non-ionic detergent, was applied to the ROS assay system. The precision and robustness of the mROS assay were evaluated by repeated measurement and calculation of *Z′*-factor, a parameter reflecting the quality of the assay. To verify the utility of the mROS assay, the number of evaluable compounds and the predictability for photosafety were compared between the ROS and mROS assays using 65 phototoxins and 18 non-phototoxic compounds.

2. Materials and methods

2.1. Chemicals

Amlodipine besylate (>98%; **5**), chlorpromazine HCl (>99%; **12**), ciprofloxacin (>98%; **14**), fenofibrate (>98%; **19**), fluvastatin Na (>98%; **21**), glibenclamide (>98%; **23**), gliclazide (>98%; **24**), griseofulvin (>95%; **25**), hydrochlorothiazide (>98%; **26**), ibuprofen (>98.5%; **27**), indomethacin (>98%; **28**), ketoprofen (>98%; **29**), lomefloxacin HCl (>98%; **31**), lovastatin (>95%; **33**), meloxicam (>98%; **35**), methotrexate (>98%; **36**), 6-methylcoumarin (>99%; **38**), mequitazine (>98%; **39**), nicardipine HCl (>99%; **42**), nitrendipine (>98%; **43**), norfloxacin (>98%; **44**), ofloxacin (>98%; **45**), omeprazole (>98%; **46**), piroxicam (>97%; **49**), pitavastatin Ca (>98%; **50**), pravastatin Na (>98%; **51**), promethazine HCl (>98%; **53**), anthracene (>99.5%; **62**), erythromycin (>98%; **68**), penicillin G (**69**), phenytoin (>98%; **70**), cinnamic acid (>99.5%; **74**), L-histidine (>98%; **76**), octrizole (>97%; **78**), *p*-aminobenzoic acid (PABA, 99.5–100.2; **81**), SDS (>99%; **82**), dimethyl sulfoxide (DMSO, >98%), imidazole (>98%), nitroblue tetrazolium (NBT, >98%), *p*-nitrosodimethylaniline (>97%), Tween 20 (2.2% water included), disodium hydrogen phosphate 12-water (>99%) and sodium dihydrogen phosphate dihydrate (>99%) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Rosiglitazone (>97%; **55**) and 4-methoxybenzylidene camphor (>99%; **77**) were purchased from Enzo Life Sciences International (Farmingdale, NY, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. Acridine HCl (>98%; **2**), bezafibrate (>98%; **9**), cilnidipine (>98%; **13**), clofibrate (>98%; **15**), naproxen (>99%; **41**), valsartan (>98%; **61**), avobenzone (>98%; **63**), hexachlorophene (>98%; **65**), benzocaine (>99%; **67**), sulisobenzone (>98%; **71**), octyl methoxycinnamate (>96%; **79**) and octyl salicylate (>98%; **80**) were bought from Tokyo Chemical Industry (Tokyo, Japan). Acridine (>97%; **1**), amiodarone HCl (>98%; **3**), benzbromarone (>95%; **8**), bufexamac (>98%; **10**), diclofenac Na (>98%; **16**), doxycycline HCl (>97%; **17**), fluphenazine 2HCl (>98%; **20**), furosemide (>98%; **22**), levofloxacin (>98%; **30**), 8-methoxypsoralen (>99%; **37**), nalidixic acid (>98%; **40**), perphenazine (**47**), quinine HCl (>99%; **54**), sparfloxacin (>98%; **56**), tamoxifen (>99%; **57**), tetracycline HCl (>95%; **58**), thioridazine HCl (>99%; **59**), bithionol (**64**), aspirin (>99%; **66**), bumetrizole (98%; **72**), chlorhexidine (>99.5%; **73**), drometrizole (97%; **75**) and UV-571 (>98%; **83**) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Amlodipine (>98%; **4**), atorvastatin (>99%; **6**), candesartan cilexetil (>99%; **11**), enoxacin (>98%; **18**), losartan K (>98%; **32**) and manidipine HCl (>99%; **34**) were obtained from LKT Laboratories (St. Paul, MN, USA). Benidipine HCl (>98%; **7**) was bought from Toronto Research Chemicals (Toronto, Ontario, Canada). Prochlorperazine dimaleate (>99%; **52**) and trifluoperazine (>99%; **60**) were purchased from MP Biomedicals (Santa Ana, CA, USA). Pirfenidone (>99%; **48**) was kindly provided by Shionogi (Osaka, Japan).

2.2. Irradiation conditions

Chemicals were stored in an Atlas Suntest CPS + solar simulator (Atlas Material Technology LLC, Chicago, 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 that of natural daylight, and the Atlas Suntest CPS series had a high irradiance capability that met CIE85/1989 daylight simulation requirements. The irradiation test was carried out at 25 °C for 1 h with an irradiance of ca. 2.0 mW/cm^2 as determined using the calibrated UVA detector Dr. Hönle #0037 (Dr. Hönle, Munich, Germany).

2.3. Reactive oxygen species (ROS) assay

ROS assay was carried out for the detection of both singlet oxygen and superoxide generation as we reported previously (Onoue et al., 2008a; Onoue and Tsuda, 2006). Briefly, each tested compound was dissolved in DMSO at 10 mM for stock solution. To monitor the generation of singlet oxygen, samples containing compounds (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) in 20 mM sodium phosphate buffer (NaPB, pH 7.4) were irradiated with simulated sunlight, and then the UV absorption at 440 nm was measured using SAFIRE (TECAN, Männedorf, Switzerland). For the determination of superoxide generation, samples containing the compounds (200 μM) and NBT (50 μM) in 20 mM NaPB (pH 7.4) were exposed to simulated sunlight, and the reduction of NBT was measured by the increase in the absorbance at 560 nm using SAFIRE. All samples were checked for precipitation by visual observation before and after light exposure. If the tested chemical was found to be insoluble in assay buffer, the assay could be carried out under appropriate dilution.

2.4. Micellar ROS (mROS) assay

Micellar solution of 0.5% (v/v) Tween 20 was applied to the ROS assay system. Critical micelle concentration (CMC) of Tween 20 was ca. 0.005% (v/v) in distilled water (Wan and Lee, 1974), and the applied concentration of Tween 20 for the mROS assay was almost identical to 100-fold of the CMC, offering high solubilizing potency. Briefly, to monitor the generation of singlet oxygen, compounds (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) were dissolved in 20 mM NaPB (pH 7.4) with 0.5% (v/v) Tween 20. For the determination of superoxide generation, compounds (200 μM) and NBT (50 μM) were dissolved in 20 mM NaPB (pH 7.4) with 0.5% (v/v) Tween 20. Then, these samples were irradiated with simulated sunlight and measured in the same conditions with the ROS assay protocol. All samples were checked for precipitation by visual observation before and after light exposure. According to the results from preliminary study, Tween 20 was found to be weak ROS generator, so results were calculated by subtracting blank readings from sample readings.