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Genotoxicity of pyrroloquinoline quinone (PQQ) disodium salt (BioPQQ™)

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ABSTRACT

The genotoxic potential of pyrroloquinoline quinone (PQQ) disodium salt (BioPQQ™) was evaluated in a battery of genotoxicity tests. The results of the bacterial mutation assay (Ames test) were negative. Weak positive results were obtained in 2 separate *in vitro* chromosomal aberration test in Chinese hamster lung (CHL) fibroblasts. Upon testing in an *in vitro* chromosomal aberration test in human peripheral blood lymphocytes, no genotoxic activity of PQQ was noted. In the *in vivo* micronucleus assay in mice, PQQ at doses up to 2000 mg/kg body weight demonstrated that no genotoxic effects are expressed *in vivo* in bone marrow erythrocytes. The weak responses in the *in vitro* test CHL cells were considered of little relevance under conditions of likely human exposure. PQQ disodium was concluded to have no genotoxic activity *in vivo*.

1. Introduction

Pyrroloquinoline quinone (PQQ) is a quinone compound initially isolated from microorganisms as a coenzyme (Salisbury et al., 1979; Duine and Jongegan, 1989). PQQ can be detected in foods at concentrations as high as 7.02 ng/g (Noji et al., 2007), and in the body, including human milk (Kumazawa et al., 1992, 1995; Mitchell et al., 1999). PQQ is an essential nutrient, and plays an important nutritional role, supporting growth and protection of cells under conditions of oxidative stress by acting as an antioxidant (reviewed in Misra et al., 2012). PQQ is a potent neuroprotective nutrient against 6-hydroxydopamine-induced neurotoxicity (Hara et al., 2007; Nunome et al., 2008), methyl mercury-induced neurotoxicity (Zhang et al., 2009a,b), *N*-methyl-D-aspartate (NMDA) receptor neurotoxicity (Zhang et al., 2008), and aggregated β-amyloid-induced toxicity (Hara et al., 2007). PQQ also has been reported to promote synthesis of nerve growth factor (NGF) in human fibroblasts (Yamaguchi et al., 1993). In addition, in rats and mice, PQQ appears to improve indices of perinatal development, possibly through increased mitochondrial content (Steinberg et al., 1994, 2003; Bauerly et al., 2006). PQQ also has been associated with preservation of mitochondrial function, through reduction of free radicals and oxidative stress (Tsuchida et al., 1993; Urakami et al., 1997; Zhu et al., 2004; Tao et al., 2007).

In light of its beneficial physiological effects, PQQ has been identified as a potential candidate for use in dietary supplements or food products for human consumption. Dietary supplements containing PQQ disodium salt (BioPQQ™) are currently commercially available in the United States (US) for use by healthy adults at a maximum serving level of 50 mg/day to maintain cognitive function, mitochondrial biogenesis, and to provide antioxidant activity. PQQ disodium salt is currently authorized in Canada as a Natural Health Product, providing 20 mg PQQ disodium salt/day as an antioxidant for the maintenance of good health (Health Canada, 2012).

While PQQ is an essential nutrient and a promising therapeutic agent, traditional safety data are lacking to support its potential use as a dietary ingredient. Beyond safety data that can be gleaned from the various rat and mouse models used to delineate the potential beneficial effects of PQQ, especially on mitochondrial function/genesis, organoprotective, and antioxidant type properties (Steinberg et al., 1994, 2003; Zhu et al., 2004, 2006; Bauerly et al., 2006, 2011; Tchaparian et al., 2010; Xiong et al., 2011), there is one report of nephrotoxicity in rats exposed by intraperitoneal injection for four days at a dose of 11.5 mg/kg body weight (bw)/

Abbreviations: 2AA, 2-aminoanthracene; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; B[a]P, benzo[a]pyrene; bw, body weight; CHL, Chinese hamster lung; DMSO, dimethyl sulfoxide; GSH, glutathione; ICH, International Conference on Harmonization; ICR-191, -methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino] acridine 2HCl; MI, mitotic index; MMC, mitomycin C; MMS, methyl methanesulfonate; NMDA, *N*-methyl-D-aspartate; NaN_3, sodium azide; NGF, nerve growth factor; PQQ, pyrroloquinoline quinone disodium salt; rpm, revolutions per minute; US, United States.

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day (Watanabe et al., 1989). In a recent study, the potential renal toxicity of PQQ disodium salt when administered orally to Sprague–Dawley rats at a dose of up to 700 mg/kg bw/day for 28 days followed by a four-week recovery period only evoked minor issues such as proteinaceous urine which recovered following termination of intake (Nakano et al., submitted for publication).

Currently, there is no data available regarding the potential genotoxic activity of PQQ disodium salt. While endogenous, and not likely to show genotoxic potential, such data are traditionally required to support the safety of a substance for use as a food ingredient for human consumption. Genetic toxicity testing of PQQ is particularly important since it is known that certain quinones (Chesis et al., 1984; Krivobok et al., 1992; Hakura et al., 1994, 1995, 1996; Cunningham et al., 1998; Lebedev et al., 2001; Kirkland and Marzin, 2003) and quinolines (Min et al., 1992; Smith et al., 1997; Chatterjee et al., 1998; Saeki et al., 2000) are active in the Ames assay, with strains TA102 and TA104 appearing to be the most sensitive, although mutagenic responses are also noted with standard OECD tester strains TA98, and TA100.

In the present study, the mutagenic and genotoxic potential of PQQ disodium salt was evaluated in a battery of genotoxicity assays including a bacterial reverse mutation test, *in vitro* chromosomal aberration tests in Chinese hamster lung (CHL) and human peripheral blood lymphocytes, and an *in vivo* micronucleus test in mice.

2. Materials and methods

All studies were conducted in compliance with Good Laboratory Practice Regulations (Ordinance No. 21, Ministry of Health and Welfare, Japan, March 26, 1997).

2.1. Chemicals and reagents

PQQ disodium salt used in all the mutagenicity and genotoxicity assays described herein was provided by Mitsubishi Gas Chemical Co. Inc. (Chiyoda-ku, Tokyo, Japan), and prepared by methods described previously by Urakami et al. (1992, 1993). 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), 2-aminoanthracene (2AA), benzo[a]pyrene (B[a]P), dimethyl sulfoxide (DMSO), sodium azide (NaN_3), and cyclophosphamide were supplied by Wako Pure Chemical Industries, Ltd., Osaka, Japan. 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino] acridine 2HCl (ICR-191) was supplied by Polysciences, Inc. PA, USA. Methyl methanesulfonate (MMS) was supplied by Tokyo Chemical Industry Co., Ltd., and mitomycin C (MMC) was supplied by Kyowa Hakko Koyo Co., Ltd., Tokyo, Japan. Phenobarbital, 5,6-benzoflavone, and S9 cofactors were supplied by Oriental Yeast Co., Ltd., Tokyo Japan. S9 fraction was purchased from Oriental Yeast Co. (Tokyo, Japan). The S9 fraction had been prepared from the liver homogenate of seven-week-old male Sprague–Dawley rats previously treated with an intraperitoneal injection with phenobarbital (daily for four days prior to sacrifice) and 5,6-benzoflavone (two days prior to sacrifice).

2.2. Bacterial reverse mutation assay (Ames test)

The bacterial reverse mutation assay was conducted in accordance with the "Guidelines for Genotoxicity Studies of Drugs" (Notification No. 1604, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare, Japan, November 1, 1999). The potential mutagenicity of PQQ disodium salt was examined in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2*uvrA*. Strains were treated with PQQ disodium salt (99.6% purity; dissolved in DMSO) at concentrations of 0 (solvent control), 10, 20, 39, 78, 156, 313, 625, 1250, 2500, or 5000 μg/plate in the absence of exogenous metabolic activation, and 0 (solvent control), 156, 313, 625, 1250, 2500, and 5000 μg/plate in the presence of exogenous metabolic activation (S9 mix) by the pre-incubation method (Maron and Ames, 1983). The solvent used was dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Doses were selected based on a preliminary range-finding test (data not shown). AF-2, NaN_3, and ICR-191 were used as positive controls in conditions without S9 mix, and B[a]P and 2AA were used as positive controls in conditions with S9 mix. All plates were incubated at 37 °C for 48 h, and the number of revertant colonies was counted with a Colony Analyzer CA-7 II (System Science Co., Ltd.). Plates were prepared in duplicate. The results were determined to be positive if the number of revertant colonies on the test plates doubled in comparison to the solvent negative control, if there was an observed dose response, and if results were reproducible. Growth inhibition was assessed through stereoscopic microscope examination of the background lawn. Statistical analyses were not conducted for this assay.

2.3. In vitro chromosomal aberration test in cultured mammalian cells

Two *in vitro* chromosomal aberration tests were conducted. In both studies, cell suspensions of lung fibroblast derived from newborn female Chinese hamsters (CHL/IU) (Laboratory Products, Department of Dainippon Pharmaceutical Co., Ltd.) were treated with PQQ disodium salt (99.3–99.7% purity; dissolved in distilled water) at concentrations of 0 (water negative control), 12.5, 25, 50, 100, 200, or 400 μg/mL, and incubated at 37 °C and 5% CO_2 for 6 and 24 h in the absence of metabolic activation (*i.e.*, the "direct method"). Cells also were treated with 0 (water negative control), 117.2, 234.4, 468.8, 937.5, 1875, or 3750 μg/mL, and incubated at 37 °C and 5% CO_2 for 6 h in the presence of S9 mix (*i.e.*, the "metabolic activation method"). Maximum concentrations of PQQ disodium salt tested were selected based on a preliminary range-finding test (data not shown) in which concentrations of 400 μg/mL (absence of S9) and 3750 μg/mL (presence of S9) were demonstrated to inhibit cell growth by 50% or greater. The dose of 3750 μg/mL is equivalent to 10 mM, the generally recommended maximum for dosing in *in vitro* assays (Galloway et al., 2011). It also is at or just above the limit of solubility of PQQ in water. MMC at 0.12 and 0.06 μg/mL was used as the positive control for plates incubated for 6 or 24 h in the absence of S9 mix, respectively; and B[a]P at 30 μg/mL was used as positive controls for plates tested in the presence of S9 mix for 6 h. Test plates were prepared in duplicate. Additional satellite groups treated with the same concentrations of the test article were included to assess cell growth inhibition (one plate per dose). Growth rate was determined as a percentage of the negative control group. The growth rate was based on the mitotic index which calculated as the percentage of 1000 total cells in metaphase. While measurement of MI provides only an indirect measure of cytotoxicity, it is acceptable for cell suspensions of this type (OECD, 1997).

Two hours prior to the completion of the incubation period, 0.2 μg/mL of colcemid solution (Demecolcine Solution, Wako Pure Chemical Industries, Ltd.) was added to each plate to arrest cells in metaphase. After the completion of the incubation period, cells were harvested with 0.25% trypsin and centrifuged and treated with 0.075 M KCl solution and 1 mL of cold fixative (3:1 methanol:acetic acid solution). Cell suspensions were pipette onto glass slides, air dried overnight, and stained with 1.25% Giemsa solution (in Sörensen buffer; 1/15 M).

Slides were evaluated by assessors blind to treatment. Chromosomal aberrations were identified either as structural aberrations or numerical aberrations (polyploidy). Structural aberrations were further classified as gaps, chromatid breaks, chromatid exchanges,