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Evaluation of the potential in vivo genotoxicity of quercetin

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ABSTRACT

Quercetin, a naturally occurring flavonol commonly detected in apples, cranberries, blueberries, and onions, has been reported to possess antioxidant, anti-carcinogenic, anti-inflammatory, and cardioprotective properties. While positive results have been consistently reported in numerous in vitro mutagenicity and genotoxicity assays of quercetin, tested in vivo, quercetin has generally produced negative results in such studies. Furthermore, no evidence of carcinogenicity related to the oral administration of quercetin was observed in chronic rodent assays. In order to further define the in vivo genotoxic potential of quercetin, a bone marrow micronucleus assay and an unscheduled DNA synthesis (UDS) assay were conducted in Wistar rats. Administered orally to male rats at dose levels of up to 2000 mg/kg body weight, quercetin did not increase the number of micronucleated polychromatic erythrocytes (MN-PCE) 24 or 48 h following dosing in the micronucleus assay. Likewise, orally administered quercetin (up to 2000 mg/kg body weight) did not induce UDS in hepatocytes of male or female rats. While measurable levels of metabolized quercetin were observed in rat plasma samples for up to 48 h after dosing, peaking at 1 h following treatment administration, the unmetabolized aglycone was not identified in either plasma or bone marrow. With the exception of only a few rats, the aglycone was also not detected in liver tissue. These results demonstrate that quercetin is not genotoxic under the conditions of these assays and further support the negative results of previously conducted in vivo assays.

1. Introduction

Flavonols are biologically and pharmacologically active polyphenolic flavonoid compounds that occur naturally in plants, where they are involved in energy production and exhibit strong antioxidant properties [1–2]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), only one of several dietary flavonols, has been identified to occur naturally in apples, cranberries, blueberries, and onions at relatively high concentrations [3–4]. Although quercetin is usually found to be conjugated with a sugar molecule in plants, purified forms of the aglycone can be obtained from natural sources *via* extraction of the glycosides and subsequent hydrolysis to release the aglycone. Many biological and pharmacological activities that may be beneficial to human health have been attributed to quercetin, including antioxidant, anticarcinogenic, anti-inflammatory, and cardioprotective activities [5–6]. While the precise mechanism or mechanisms of action that underlie these properties have yet to be fully elucidated, ongoing research in this area seems to indicate that many of these activities are a function of the antioxidant actions of the flavonol [7–10]; however, as with many other antioxidants (e.g., vitamin C), the generally favorable antioxidant effects of quercetin have been closely linked with the potential generation of reactive pro-oxidant intermediates [11–12]. Thus, it is not surprising that quercetin has also been shown to be a consistent in vitro mutagen and genotoxicant.

In order to assess its mutagenic and genotoxic potential, quercetin has been extensively tested under in vitro and in vivo experimental conditions. When tested in vitro in most standard strains of *Salmonella typhimurium,* quercetin has consistently displayed positive mutagenic activity, irrespective of

Abbreviations: 2-AAF, 2-acetylaminofluorene; $Al(NO_3)_3$, aluminium nitrate; CAS, Chemical Abstracts Service; CH_3CN, acetonitrile; CGC, cytoplasmic grain counts; CPA, cyclophosphamide; DMN, dimethylnitrosamine; DNA, deoxyribonucleic acid; HPLC, high-performance liquid chromatography; HPMC, hydroxypropylmethylcellulose; KH_2PO_4, potassium phosphate; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; LOD, limit of detection; MN, micronuclei; MN-PCE, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocytes; NCE:PCE, ratio of normochromatic erythrocytes to polychromatic erythrocytes; NGC, nuclear grain counts; NNG, net nuclear grains; OECD, Organisation for Economic Co-Operation and Development; PCE, polychromatic erythrocytes; TBHQ, *tert*-butylhydroquinone; UDS, unscheduled DNA synthesis.

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metabolic activation [13–31]. Similarly, in *Escherichia coli*, quercetin has been shown to induce SOS activity, reverse mutations, and DNA single strand breaks with or without metabolic activation [14,23,30,32–35]. These positive mutagenic and genotoxic responses in bacterial test systems have been confirmed in eukaryotic cells, including yeast [14] and mammalian cells (i.e., mouse, hamster, rat, and human) [23,29,36–44], in which sister chromatid exchanges, chromosomal aberrations, unscheduled DNA synthesis (UDS), and micronucleus formation were among some of the endpoints evaluated. Conversely, plasma samples obtained from rats treated orally with quercetin did not exhibit any mutagenic properties when tested in *S. typhimurium* [25]. Furthermore, studies examining genotoxic endpoints, including endpoints similar to those evaluated in vitro (e.g., micronucleus formation, chromosomal aberrations, sister chromatid exchange, UDS, and alkali-labile DNA damage) in mice and rats following single and repeat oral administration of quercetin have shown consistently that, in spite of its positive in vitro activity, quercetin does not exert mutagenic or genotoxic properties in vivo [45–50]. The negative results of the in vivo genotoxicity studies are further corroborated by numerous toxicological studies including a 2-year rodent carcinogenicity bioassay conducted by the National Toxicology Program (NTP), which have not presented any adverse effects that would be relevant in an assessment of quercetin safety for oral consumption in humans [29,51–53]. Although the NTP study initially concluded some evidence of carcinogenicity in male rats related to increased incidences of renal tumors [29], this was subsequently shown to be mediated by the exacerbation of chronic nephropathy (i.e., increased incidences of tumor types known to be associated with late-stage chronic progressive nephropathy in the male rat), rather than by the direct action of quercetin [53].

The purpose of this study was to further assess the in vivo genotoxic potential of quercetin by examining the possibility of micronucleus formation in bone marrow cells and UDS in hepatocytes of Wistar rats treated with quercetin at dose levels at least 2-fold greater than previously tested in either assay [45–46,48]. Additionally, unlike in the previous UDS assay in which only gastric mucosal cells were evaluated [48], UDS in the present study was examined in hepatocytes exposed to quercetin and its metabolites following absorption.

2. Materials and methods

2.1. Chemicals

Quercetin (CAS Registry No. 117-39-5) (Lot No. K91027842) used in both assays was provided by Merck KGaA (Darmstadt, Germany). The purity of the test material was reported to be 103.6% (on dry weight basis; maximum 10% water so adjusted to 93.6%). In the micronucleus assay, quercetin was suspended in 10 mL of a 0.25% aqueous solution of hydroxypropylmethylcellulose (HPMC) (Methocel^R K4M Premium; Colorcon, Dartford, Kent, UK) to a final concentration of 200 mg quercetin/mL and was serially diluted to obtain lower concentrations. The solvent also was used as the negative control. The positive control, cyclophosphamide (CPA) (Endoxan^R), was obtained from Asta (Frankfurt, Germany) and was dissolved in 10 mL of "water for injection" directly before administration to a final concentration of 1.65 mg CPA/mL.

For the UDS assay, quercetin was suspended in a 0.5% aqueous HPMC solution (Methocel^R K4M Premium; Colorcon, Dartford, Kent, UK). As in the micronucleus assay, HPMC served as the negative control. Positive controls, 2-acetylaminofluorene (2-AAF) and dimethylnitrosamine (DMN), were obtained from Sigma Chemical Co. (Poole, UK). 2-AAF was dissolved in corn oil to a final concentration of 7.5 mg/mL, while DMN was dissolved in purified water to a final concentration of 1.0 mg/mL.

2.2. Animals

For the micronucleus assay, 60 male Wistar HsdCpb:WU rats were obtained from Harlan Winkelmann (Borchen, Germany) at 7–9 weeks of age (weighing 232.1–265.8 g) and acclimated to the laboratory conditions for 7 days prior to initiation of the study. The rats were housed individually in Makrolon cages (Type 3) on softwood chippings under conventional environmental conditions in artificial light (i.e., 55–72% atmospheric humidity; 21–23 °C; 759–763 mm Hg atmospheric pressure; 12-h light:12-h dark cycle). A standard laboratory pellet diet (Altromin TPF^{R}N 1324; Chr. Petersen A/S, Ringsted, Denmark) and tap water from Makrolon drinking bottles were provided *ad libitum* throughout the experimental period.

Table 1
Control and test groups used in the micronucleus test[a]

Group no.	Test or control article	Dose (mg/kg bw)	Observation period (h)
1	0.25% HPMC (negative control)	–	24
2	Quercetin	200	24
3	Quercetin	632	24
4	Quercetin	2000	24
5	Quercetin	2000	48
6	CPA (positive control)	16.5	24

HPMC, hydroxypropylmethylcellulose; CPA, cyclophosphamide.
[a] Route of administration: oral; dosing volume: 10 mL/kg bw; 5 animals/group.

For the UDS assay, 88 male and 88 female Wistar (Out-bred Han Wistar Crl:Wi[Han]) rats were obtained from Charles River Laboratories Ltd., UK (Margate, UK) at 5–6 weeks of age (with body weights of 201–259 g for males and 169–218 g for females) and acclimated for 6–9 days to laboratory conditions before testing was initiated. The rats were housed in polypropylene cages with wire mesh lids and solid floors in groups of no more than 3 rats per cage under the following conditions: 12-h light: 12-h dark cycle with artificial light; 15 fresh air changes per hour; 20–22 °C; and 52–66% humidity. A standard laboratory pellet diet provided by Special Diet Services Ltd. (RM1 [E] SQC; Witham, Essex, UK) and tap water from Makrolon drinking bottles were provided *ad libitum* throughout the experimental period.

2.3. Experimental procedure

2.3.1. Micronucleus assay[1]

The micronucleus assay was conducted in compliance with the Commission Directive 92/69/EEC [54] and the Organisation for Economic Co-Operation and Development (OECD) Guideline for Testing of Chemicals No. 474 [55].

A preliminary dose-finding study was conducted before the start of the micronucleus assay, in which 15 male rats were administered quercetin once at a dose level of 2000 mg/kg body weight and subsequently observed for 48 h for any signs of toxicity. Since quercetin-treated rats did not exhibit any signs of toxicity, 2000 mg/kg body weight was chosen as the highest dose level for the micronucleus assay.

Following the acclimatization period, 30 animals were randomly divided into 6 groups of 5 rats (Table 1). Dosing was conducted over 1–3-day intervals to allow for sufficient sample preparation time. Quercetin was administered to test animals as a single oral dose at levels of 200, 632, or 2000 mg/kg body weight by gavage using a stomach tube and a disposable syringe. Apart from the quercetin test groups, 2 other groups (5 rats/group) received the solvent solution (0.25% HPMC; negative control) or CPA (16.5 mg/kg body weight; positive control) by oral gavage. Following treatment administration, animals were observed for clinical signs of toxicity for a period of 24 h. Additionally, a further group of 5 animals was administered 2000 mg quercetin/kg body weight and maintained for observation for 48 h. Body weights were recorded at the end of the observation period or daily in the case of the rats observed for 48 h. The test articles were administered at a dosing volume of 10 mL/kg body weight. Body weights obtained prior to dosing were used to calculate the actual volume of administration.

At the end of the observation period, the animals were killed by carbon dioxide asphyxiation. Immediately after death, 1 femur from each animal was dissected and cleaned from adherent muscles. The epiphyses were cut off and bone marrow cells were flushed out with foetal calf serum (Biochrom, Berlin, Germany). The suspension of bone marrow cells and foetal calf serum was filtered through cellulose according to Romagna [56] and centrifuged for 5 min at 150 × *g*. The resulting sediment was resuspended in foetal calf serum. Bone marrow smears were prepared from the resulting cell suspension and stained according to a modified Giemsa-staining method described by Gollapudi and Kamra [57] using Giemsa's solution with Weise buffer solution and mounted in Entellan (Merck, Darmstadt, Germany).

The slides were analyzed in a blinded fashion using a Zeiss light microscope with plane optics. Both normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) were scored for micronuclei (MN) and counted separately up to a total of 1000 erythrocytes per animal. Thereafter, counting was limited to PCE only, for a total of 2000 PCE per animal. The number of NCE counted differed for every animal, ranging from 424 to 715 NCE per animal (mean 562 ± 69). For every group of animals, the following parameters were reported: the number of PCE; the number of MN-containing cells/1000 PCE/animal; the number of NCE; the number of MN-containing cells/1000 NCE/animal; and the NCE:PCE ratio. The number of MN-

[1] Studies were conducted at Merck KGaA, Institute of Toxicology and Central Services Analysis (64271 Darmstadt, Federal Republic of Germany).