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Mutagenic potential of the isoflavone irilone in cultured V79 cells

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HIGHLIGHTS

- The isoflavone irilone was tested in cultured Chinese hamster lung fibroblast V79, and . . .
- Inhibited proliferation and did not increase the mutant frequency in the HPRT assay.
- Altered significantly the mutation spectrum.
- Significantly increased micronuclei with chromosomal fragments.
- Disrupted mitosis and significantly increased micronuclei with whole chromosomes.

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ABSTRACT

After consumption of red clover-based dietary supplements, plasma concentrations of the isoflavone irilone (IRI) equal that of the well-investigated daidzein. Since some isoflavones are genotoxic, the potential of IRI to induce mutations was investigated.

Gene mutations were determined by hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay and sequencing of mutant cDNA, chromosome and genome mutations by micronucleus assay complemented by immunochemical staining of centromere proteins and microtubules in cultured V79 cells. Cell proliferation was monitored by electronic cell counting, flow cytometry and fluorescence microscopy.

IRI did not affect the mutant frequency in the *Hprt* locus but altered the mutation spectrum by increasing the proportion of deletions and decreasing that of base pair substitutions. Induction of chromosome mutations was supported by a slight but significant increase in the number of micronucleated cells containing chromosomal fragments despite activation of three cell cycle checkpoints possibly interfering with micronuclei formation. Moreover, IRI exhibited a strong aneugenic potential characterized by disrupted mitotic spindles, mitotic arrest, and asymmetrical cell divisions leading to chromosome loss, nuclear fragmentation as well as mitotic catastrophe. Thus, IRI might be another isoflavone to be taken into account in safety assessment of dietary supplements.

1. Introduction

The isoflavone irilone (IRI, Fig. 1) was first isolated in 1973 from rhizomes of *Iris germanica* (Dhar and Kalla, 1973). In the following years, IRI was also detected in other plant species including red clover (Wu et al., 2003) and kudzu (Mansoor et al., 2011), the extracts of which are used in dietary supplements.

Data on the IRI content in red clover based dietary supplements are scarce but suggest that in relation to the main red clover isoflavones formononetin and biochanin A aglycone and glycoside IRI accounts for about 3–14% and 5–18%, respectively (Lutter et al., 2014). A human intervention pilot study with red clover dietary supplements revealed IRI plasma levels of 0.2–0.6 μM, which was equal or higher than those of formononetin and biochanin A and their metabolites daidzein and genistein (Maul and Kulling, 2010). Despite the high levels reached in human plasma information on the biological effects of IRI in mammalian cells is scarce. IRI was estrogenic in two cultured human cell lines (Lutter et al., 2014). Starting at slightly cytotoxic concentrations, IRI induced NAD(P)H: quinone reductase activity 2-fold in cultured mouse hepatoma cells (Wollenweber et al., 2003), and induced apoptosis in cultured human hepatoma cells (Mansoor et al., 2011).

Abbreviations: 6-TG, 6-thioguanine; DAPI, 4′,6-diamidino-2-phenylindole; DES, diethylstilbestrol; EMS, ethyl methanesulfonate; ETO, etoposide; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IRI, irilone; MMC, mitomycin C; NQO, 4-nitroquinoline *N*-oxide; PE, plating efficiency.

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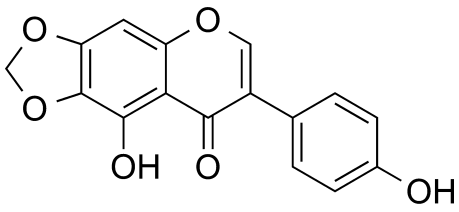

Fig. 1. Chemical structure of IRI.

Information on the mutagenicity and genotoxicity of IRI is lacking completely. Isoflavones have been known to cause gene mutations (Tsutsui et al., 2003) and to induce micronuclei (Kulling et al., 2002) in cultured mammalian cells. Thus, the ability of IRI to induce mutations at the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene locus and to induce micronuclei in Chinese hamster V79 lung fibroblast was investigated. In addition, cDNA sequencing of mutant colonies was performed to allow the molecular characterization of the mutant phenotype.

2. Materials and methods

2.1. Chemicals

IRI (purity >95%) was obtained from ChromaDex™, Irvine, CA, USA) and diethylstilbestrol (DES, >98%) from Biomol, Germany. Mitomycin C (MMC, ≥98%), etoposide (ETO, ≥98%), ethyl methanesulfonate (EMS), 4-nitroquinoline *N*-oxide (NQO, ≥98%), and DMSO (99.9%) were obtained from Sigma–Aldrich, Germany. PCR reagents and equipment were obtained from Applied Biosystems, Germany, and all other chemicals, cell culture media and medium supplements were obtained from Sigma–Aldrich or Roth, Germany unless specified otherwise.

2.2. Cell culture conditions

In all experiments, the well-characterized V79 Chinese hamster fibroblast cell line (Bradley et al., 1981) was used which does not express any cytochrome P450-dependent monooxygenases, uridine diphosphate glucuronosyltransferases (Glatt et al., 1987) or sulfotransferases (Glatt et al., 2000) but has detectable activity of soluble catechol-*O*-methyl transferase (118 ± 51 pmol quercetin/min/mg cytosolic protein; unpublished data) and glutathione-*S*-transferase activity (Glatt et al., 1987). V79 MZ cells, kindly provided by H. Glatt (German Institute of Human Nutrition, Potsdam, Germany), were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Invitrogen™ Life Technologies, Germany) referred to as DMEM complete.

2.3. Hprt *mutant frequency and mutational spectrum*

2.3.1. HPRT assay

The HPRT assay has been performed as described previously (Schumacher et al., 2005) with slight modifications. 1.5×10^6 V79 cells were seeded in cell culture flasks (175 cm^2, Greiner Bio-one, Germany) containing 20 ml DMEM complete. After 24 h, the medium was changed (day 0) and cells were treated with different concentrations of IRI or 1 μM NQO or 1% DMSO for 24 h. A total of 1×10^6 treated cells were subcultured in fresh medium directly after treatment (day 1) and again on day 4. On days 1, 4, and 6, the number of viable cells and cells with disintegrated cell membrane were counted with an electronic cell counter (CASY Model DT, Schaerfe System GmbH, Germany) as a measure for cytotoxicity and proliferation. On day 6, cells with mutations at the *Hprt* gene locus were selected by growing cells in DMEM complete, and 7 μg 6-thioguanine (6-TG)/ml using three tissue culture dishes (145 mm, Greiner Bio-one) with 1×10^6 cells per dish. To determine the plating efficiency (PE) on days 1 and 6, cells were grown in the absence of 6-TG (500 cells per 100 mm dish, three dishes). After 1 week, cells were fixed with ethanol and stained with methylene blue. Colonies were counted and the PE, i.e., the number of colonies per number of seeded cells, and the mutant frequency, i.e., the number of colonies/(number of seeded cells × the PE at day 6), were calculated.

2.3.2. Cloning and cDNA preparation

For cDNA sequencing of 6-TG-resistant mutants were cloned using cloning rings, total RNA was isolated, and reversely transcribed (using oligo-(dT) 18 primer) as detailed in Scheffler et al. (2015).

2.3.3. Amplification, isolation and sequencing of V79 cDNA

Reverse primer 5′-ATGAACTGAGTGCTTTCACA-3′ (exact positions depicted in Fig. 3 in Scheffler et al. (2015)) and forward primer 5′-CTTCCTCCTCACACCGCTCT-3′ were checked for lack of secondary structures and unspecific amplification products by PrimerExpress 3.0-licensed-(Applied Biosystems). Positioning of primers in the 3′- and 5′-untranslated region of *Hprt* cDNA, respectively, ensured reliable sequencing and duplicate determination of all exons (Fig. 3 in Scheffler et al. (2015)). 600 ng *Hprt* cDNA dissolved in nuclease free water and 4 μl forward or reverse primer (5 μM), respectively were mixed in a volume of 14 μl and subjected to Sanger sequencing by LGC Genomics, Germany.

2.3.4. Analysis of sequencing data and of mutational spectra

Resulting electropherograms were analyzed using Chromas Lite (freely available at http://www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml) and resulting sequences were compared using ApE (freely available at http://biologylabs.utah.edu/jorgensen/wayned/ape/) and Referenz Sequenz cDNA: GenBank: J00060.1; http://www.ncbi.nlm.nih.gov/nuccore/J00060.1. type and position of mutations within the cDNA were determined by sequence alignment.

2.4. Micronucleus assay

2.4.1. Cell exposure

V79 cells were grown on chamber slides (Thermo Fisher Scientific Inc., Germany) with eight chambers per slide (5–7×10^3 cells per cm^2) for 24 h prior to incubation with IRI, DES, ETO, MMC, and EMS or with the solvent (1% DMSO) for 6 h with and without subsequent substance-free incubation for up to 40 h.

2.4.2. Fixation and immunological staining

Following incubation with the test compounds, cells were stained as described previously (Lehmann and Metzler, 2004) with slight modifications (Scheffler et al., 2015). Briefly, cells were fixed with paraformaldehyde and methanol and stained with anti-α-tubulin (Sigma–Aldrich) for the evaluation of cell morphology and the detection of freshly divided cells, and with anti-centromere protein antibodies (from patients with limited systemic scleroderma/CREST syndrome, Antibodies Inc., Davis, CA, USA) followed by secondary Cy3-conjugated goat anti-mouse antibody (Jackson Immune Research Laboratories, Inc., West Grove, USA) and FITC-conjugated goat polyvalent anti-human antibody (Sigma–Aldrich). Finally, slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) containing antifade solution.

2.4.3. Fluorescence microscopic analysis

Slides were coded and analyzed with an Observer Z1 fluorescence microscope (Zeiss, Germany) equipped with a