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Effects of chemopreventive natural products on non-homologous end-joining DNA double-strand break repair

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ABSTRACT

Double-strand breaks (DSBs) may result from endogenous (*e.g.*, reactive oxygen species, variable (diversity) joining, meiotic exchanges, collapsed replication forks, nucleases) or exogenous (*e.g.*, ionizing radiation, chemotherapeutic agents, radiomimetic compounds) events. DSBs disrupt the integrity of DNA and failed or improper DSBs repair may lead to genomic instability and, eventually, mutations, cancer, or cell death. Non-homologous end-joining (NHEJ) is the major pathway used by higher eukaryotic cells to repair these lesions. Given the complexity of NHEJ and the number of proteins and cofactors involved, secondary metabolites from medicinal or food plants might interfere with the process, activating or inhibiting repair. Twelve natural products, arbutin, curcumin, indole-3-carbinol, and nine flavonoids (apigenin, baicalein, chalcone, epicatechin, genistein, myricetin, naringenin, quercetin, sakuranetin) were chosen for their postulated roles in cancer chemoprevention and/or treatment. The effects of these compounds on NHEJ were investigated with an *in vitro* protocol based on plasmid substrates. Plasmids were linearized by a restriction enzyme, generating cohesive ends, or by a combination of enzymes, generating incompatible ends; plasmids were then incubated with a nuclear extract prepared from normal human small-intestinal cells (FHS 74 Int), either treated with these natural products or untreated (controls). The NHEJ repair complex from nuclear extracts ligates linearized plasmids, resulting in plasmid oligomers that can be separated and quantified by on-chip microelectrophoresis. Some compounds (chalcone, epicatechin, myricetin, sakuranetin and arbutin) clearly activated NHEJ, whereas others (apigenin, baicalein and curcumin) significantly reduced the repair rate of both types of plasmid substrates. Although this *in vitro* protocol is only partly representative of the *in vivo* situation, the natural products appear to interfere with NHEJ repair and warrant further investigation.

1. Introduction

DNA double-strand breaks (DSBs) are generated when the two complementary strands of the DNA double helix are broken simultaneously at sufficiently close sites such that base-pairing and chromatin structure cannot keep the two ends juxtaposed [1–3]. DSBs, which may result from endogenous or exogenous processes, are one of the most critical DNA lesions, in terms of cell death. Cellular metabolism may lead to spontaneous DSBs, induced by reactive oxygen species, collapsed replication forks, nucleases, or by the physical stress generated when dicentric or catenated chromosomes are pulled to opposite poles during mitosis [4]. Mammalian cells suffer at least 10 spontaneous DSBs per cell cycle [5]. DSBs also constitute natural steps in programmed genomic rearrangement processes, such as variable (diversity) joining (V[D]J) recombination, class-switch recombination, and meiotic exchange. Exogenous DSBs are notably induced by ionizing radiation, chemotherapeutics (*e.g.*, topoisomerase poisons), or radiomimetic compounds [6–8].

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Consequences of misrepaired DSBs may include chromosome alterations, such as deletions, translocations or fusions, that may lead to genomic instability, premature aging, cell death, or development of cancer [4,8–10]. Efficient DSB repair mechanisms are therefore critical for cell survival and genomic integrity [5]. The repair of DSBs involves two main different pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ) [5,11]. HR is a highly accurate process that uses undamaged homologous DNA segments (sister-chromatid, homologous chromosome, or repeated regions on the same or different chromosomes) to rejoin DSBs; the original DNA sequence is therefore restored at the break. HR takes place mainly in the late S/G2 phase, when a sister chromatid is available. NHEJ joins two broken ends directly end-to-end; since NHEJ does not need a homologous DNA template, it can operate when only one chromosomal copy is available. NHEJ is therefore a major DSB repair mechanism in higher eukaryotes during the G1/early S phase of the cell cycle [8,12]. Extensive chromosome condensation in higher eukaryotic cells may make homology search extremely difficult in the G1 phase, which explains the predominant use of NHEJ by mammalian cells [9,13]; this mechanism is the focus of the present study.

The NHEJ pathway requires the concerted action of several components, including four core factors: the DNA-end-binding Ku70/Ku80 heterodimer; the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which forms a complex with the Artemis (DNA-PKcs/Artemis) nuclease; the X-ray cross-complementation 4 (XRCC4)/ligase IV complex; and the XRCC4-like factor/Cernunnos (XLF/Cer), which interacts with the (XRCC4)/ligase IV, stimulates its ligase activity [14], and quickly responds to DSB induction, accumulating at damaged sites in a Ku-dependent manner [15]. The nuclease Mre11, the polymerases (Pols λ, μ, and terminal deoxynucleotidyl transferase), the tyrosyl-DNA-phosphodiesterase 1 and the exonuclease 1 can also be involved. Given the complexity of this pathway and the large number of proteins and cofactors involved, it seems logical that exogenous compounds from food or medicinal plants may affect the process, activating or inhibiting repair [16,17].

Classical *in vitro* NHEJ assays are based on the oligomerization of linearized plasmids by protein extracts, followed by the analysis of the oligomers on agarose gel electrophoresis [5,11,18–23]. Although these tests are clearly an over-simplification of the real i*n vivo situation* (broken DNA can be joined in many ways, most simply by exonuclease digestion and/or ligation; the influence of chromatin is certainly important for these DNA repair systems), they are widely used for probing NHEJ [24–26]. Gel-based methods show limitations in resolution, reproducibility and quantification; manual and time-consuming [27,28], they are not adapted to screening for modulators of DSBs repair. In our previous work, an original NHEJ assay protocol using on-chip microelectrophoresis was devised, to allow automation of all the electrophoresis steps (separation, staining, destaining, band detection and data analysis), strongly reducing the manual and timely procedures associated with gel handling, the required amounts of reagents and samples, waste, and exposure to hazardous chemicals [29].

We are examining whether polyphenols previously suggested to play roles in cancer chemoprevention and/or treatment [30–61] (summarized in Table 1) can affect *in vitro* DSBs repair in human cells, effects which might bear on their chemopreventive properties. To this end, the potential modulatory effects of nine flavonoids, including phytochemicals particularly abundant in our diet (apigenin, baicalein, chalcone, epicatechin, genistein, myricetin, naringenin, quercetin and sakuranetin), arbutin, curcumin and indole-3-carbinol (Fig. 1, Table 1) on *in vitro* DSB repair by the NHEJ pathway were studied.

2. Material and methods

2.1. Chemicals

Quercetin dihydrate was obtained from Riedel-de-Haën laboratory (Seelze, Germany); apigenin, arbutin, baicalein, chalcone, epicatechin, indole-3-carbinol and naringenin from Sigma–Aldrich-Fluka (St. Louis, MO, USA); curcumin, genistein and myricetin from Carl Roth (Karlsruhe, Germany); and sakuranetin from Extrasynthèse (Genay, France).

Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum Gold (FBS Gold), glutamine, penicilline/streptomycine mix, non-essential amino acids, Hepes buffer and Accutase were obtained from PAA Laboratories Inc (Pasching, Austria). Adenosine triphosphate (ATP), dithiothreitol (DTT), 3(-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), epithelial growth factor, ethylene diamine tetraacetate (EDTA), glycerol, HEPES, insulin, magnesium chloride, potassium chloride, potassium hydroxide, sodium chloride, TRIS base, Triton X-100, were obtained from Sigma–Aldrich-Fluka. NU7441 was from Bioconnect (Huissen, Nederland).

The plasmid *pGEM-7Z* (3 kb) was obtained from Promega (Fitchburg, MA, USA), amplified in *Escherichia coli*, isolated and purified using QIAGEN™ Plasmid Maxiprep Kit (Hilden, Germany).

Restriction enzymes (RE), proteinase K, and ProteoBlock protease inhibitor cocktail were obtained from Fermentas laboratory (Vantaa, Finland) and used as per the manufacturer's protocol; acetic acid and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany).

The natural product compound were dissolved in DMSO and diluted with culture medium. The final concentration of DMSO in contact with cells was no more than 0.5% (v/v).

2.2. Cell culture

The small intestinal epithelial cell line, FHs 74 Int ATCC CCL-241, was obtained from LGC Standards (Molsheim, France). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, in DMEM medium supplemented with 10% FBS, 4 mM glutamine, 100,000 U/l penicillin, 100 mg/l streptomycin, 10 mM hepes buffer, 1% non-essential amino acids, 10 mg/l insulin and 30 μg/l epithelial growth factor. Cells were harvested at about 80% confluence and used between passages 27 and 30 [62].

2.3. MTT cytotoxicity testing

Cells (2×10^4 in 200 μl complete medium) were seeded in 96-well plates and grown at 37 °C for 22–26 h before a 50 μl aliquot of a base-2 logarithmic dilution of a tested compound was added (concentrations 0.8–200 μM). After a further 24 h culture, the medium was replaced by 200 μl of MTT [3(-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] solution in DPBS (0.5 mg/ml) and the plates left for a further 3.5 h in the incubator. The supernatant was replaced by DMSO to dissolve the crystals of reduced formazan (15 min agitation, 700 rpm) and the absorbances were measured with a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) at 540 nm (A_{540}) and 620 nm (A_{620}), comparing to untreated control cells. The fitted curves "% of living cells" *versus* "log (concentration of the compound)" allowed selection of the IC_{10} (the concentration that inactivates cellular growth by 10%) for subsequent experiments; if IC_{10} was >30 μM (low-toxicity compounds), this latter concentration was selected (Table 2). Steady-state plasma concentrations of flavonoids are usually not much higher than 1 μM, even in persons who consume large amounts of plant material. There is, however, evidence that flavonoids may accumulate in the cell and tissues, although the exact concentrations reached are still not known [63]. The 30 μM maximal concentration is then a compromise, to avoid non-specific effects.

2.4. Nuclear extract preparation

All experiments were performed on four biological replicates from either natural product-pretreated cells (contact, 24 h) or control cells; for each experiment, nuclear extracts (NE) were prepared in du- or triplicates, according to the protocol of Millau et al. [64].

Non-confluent cells were detached with Accutase, collected by centrifugation (4 °C, 7 min, 500 g) and washed twice in ice-cold DPBS. The cell pellet was resuspended in ice-cold buffer A (HEPES 10 mM pH 7.9, $MgCl_2$ 1.5 mM, KCl 10 mM, Triton X-100 0.01%, DTT 0.5 mM, Protease Inhibitor Cocktail 1%) to yield 2×10^6 cells/ml. Cytoplasmic membranes were lysed on ice for 10 min, and lysis was achieved by vortexing for 30 s. Nuclei were collected by centrifugation (4 °C, 10 min, 2000 g) and suspended in ice-cold buffer B (HEPES 10 mM pH 7.9, $MgCl_2$ 1.5 mM, KCl 400 mM, EDTA 0.2 mM, glycerol 25%, DTT 0.5 mM, Protease Inhibitor Cocktail 1%) to yield 2×10^6 nuclei/25 μl. Nuclei were incubated on ice for 20 min and lysed by two freezing (−80 °C)–thawing (4 °C) cycles. Extracts were cleared by centrifugation (4 °C, 15 min, 16 000 g), aliquoted and stored at −80 °C. The protein concentration was determined using the Pierce BCA Protein Assay Kit and expressed as equivalent of bovine serum albumin (Thermo Scientific, Vantaa, Finland).