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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).

Table 1
Cancer chemoprevention-related activities of investigated polyphenols.

Compound	Cancer chemoprevention related activities	References
Apigenin	Inhibits pancreatic cancer cell growth and induces apoptosis *in vitro*	[36]
	Reduces the size of xenograft tumors in a dose-dependent manner with increased cellular apoptosis	
	Exerts synergistic pro-apoptotic effects at low dose (15 μM) by suppressing superoxide dismutase activity in combination with paclitaxel	[37]
	Increases the excision of ethyl methanesulfonate-induced damages in comet assay	[67]
Baicalein	Reduces the adhesion, invasion and migration of cutaneous squamous carcinoma cells and human hepatoma cells	[38,39]
Chalcone	Has valuable potential as an antiangiogenic and anti-cancer agent	[40]
	Its action is mediated through the inhibition of multi-target Receptor Tyrosine Kinases including Vascular Endothelial Growth Factor receptor	
Epicatechin	Shows potential preventive activity for oral, prostate and colorectal cancer	[41,42]
	Stimulates the excision of ethyl methanesulfonate-induced damages	[67]
Genistein	Induces the expression of the genes BRCA1 and BRCA2 that encode proteins involved in recombinational repair	[63]
	Shows an inhibition of the initiation and promotion of chemically-induced skin tumorigenesis in animal models	[44]
	Stimulates carcinogenesis and greatly enhances metastatic capacity in prostate cancer at low, nutritionally relevant, doses	[43]
	Presents inhibitory effects on tyrosine kinase, topoisomerase II, 5-α-reductase, and angiogenesis at high concentrations	[43]
Myricetin	Stimulates the repair of oxidatively damaged DNA (global effect measured through the comet assay; no mechanism proposed so far)	[67]
	Strongly inhibits neoplastic cell transformation through the direct inhibition of a series of kinases and acts as a promising agent for the chemoprevention of skin cancer	[45,64]
	Exerts pro-apoptotic effect through a ROS-independent pathway on a human leukemia cell line	[46]
Naringenin	Enhances the repair of oxidative and UV-B induced genomic DNA damages by promoting the removal of 8-oxo-dGuo and pyrimidine dimers	[65,66]
Quercetin	Plays a role in the regulation of cell signaling, cell cycle and apoptosis but available epidemiological studies are still controversial on its possible effectiveness in cancer prevention	[68]
	Is consistently reported positive in numerous *in vitro* mutagenicity and genotoxicity assays	[48–50]
	Induces positive response by itself in the comet assay	[51]
	Is strongly clastogenic	[52]
	Extensive *in vivo* studies have not shown any evidence of quercetin genotoxicity or carcinogenicity	[53,54]
Sakuranetin	Suppresses the *Salmonella typhimurium* SOS response and exerts an antimutagenic effect in the Ames test	[69]
	Increases excision of the ethyl methanesulfonate-induced damages	[67,69]
Arbutin	Inhibits human bladder carcinoma cell proliferation *via* extracellular signal-regulated kinase inactivation and p21 up-regulation	[56]
Curcumin	Modulates many components of intracellular signaling pathways implicated in inflammation, cell proliferation and invasion and induces genetic modulations eventually leading to tumor cell death	[57–59]
	Suppresses oesophageal cancer cell growth *in vitro* and in nude mouse xenografts in combination with epigallocatechin gallate and lovastatin	
	Interferes with prostate cancer proliferation and metastasis development by down-regulating the androgen and epidermal growth factor receptors and inducing cell cycle arrest	
Indole-3-carbinol	Inhibits tumor growth *in vivo*, promotes apoptosis and interferes with genes affecting multiple pathways important for cancer progression in combination with bortezomib for the treatment of ovarian cancer	
	Inhibits telomerase activity and hTERT mRNA expression in prostate cancer cells	
	Targets multiple aspects of cancer cell cycle regulation and survival including caspase activation, cyclin-dependent kinase activities, estrogen metabolism, estrogen receptor signaling, endoplasmic reticulum stress, and BRCA gene expression	[60,61,70]

2.5. Plasmids

Double strand breaks at defined sites were created by digesting plasmid *pGEM-7Z* (3 kb), with restriction enzymes, either *Cfr*9I to generate cohesive ends, or the combination of *Apa*I and *Kpn*I to generate two types of fragments: short fragments, which were discarded; and long fragments with incompatible ends, which were separated by agarose gel electrophoresis and purified from the cut agarose band using the GenElute Gel Extraction Kit (Sigma–Aldrich-Fluka, St Louis, MO, USA).

2.6. In vitro assay of nonhomologous end-joining activity

Linearized plasmids *pGEM-7Z* (3 kb) were incubated with a nuclear extract obtained from either natural product-pretreated cells or control cells (Fig. 2). End-joining reactions were performed in 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 80 mM KCl, 1 mM ATP, 1 mM DTT, with 0.25 μg of DNA (12.5 ng/μl linearized pGEM-7Z plasmid) and 0.4 μg/μl nuclear extract (NE), obtained from either pretreated cells (24 h contact) or control cells, in a final volume of 20 μl. Mixtures were incubated at 37 °C for 20 min (linearized plasmids with cohesive ends) or at 25 °C for 60 min (linearized plasmids with incompatible ends). Reactions were ended by adding 2 μl of sodium dodecyl sulfate, 0.5% (for linearized plasmids with cohesive ends) or Triton X-100, 0.5% (for linearized plasmids with incompatible ends), 0.5 M EDTA, 2 μl, and proteinase K (10 mg/ml, 1 μl), and incubating for 60 min at 50 °C. A well-known inhibitor of DNA-PKcs, NU7441 (10 μM; 20 min nuclear extract pretreatment on ice followed by end-joining reaction) was tested as a positive control for NHEJ inhibition. End-joining reactions were only performed in DNA LoBind microtubes (Eppendorf, Hamburg, Germany).

2.7. Automated microchip electrophoresis

Particles were discarded by centrifugation (4 °C, 15 min, 16,000 g), and the supernatant was applied on Experion™ DNA 12 K chips and electrophoresed on the Automated microchips electrophoresis Experion™ system (BioRad Laboratories, Hercules, CA, USA).

The data were reported as electropherograms, simulated gel images, and tabulated formats.

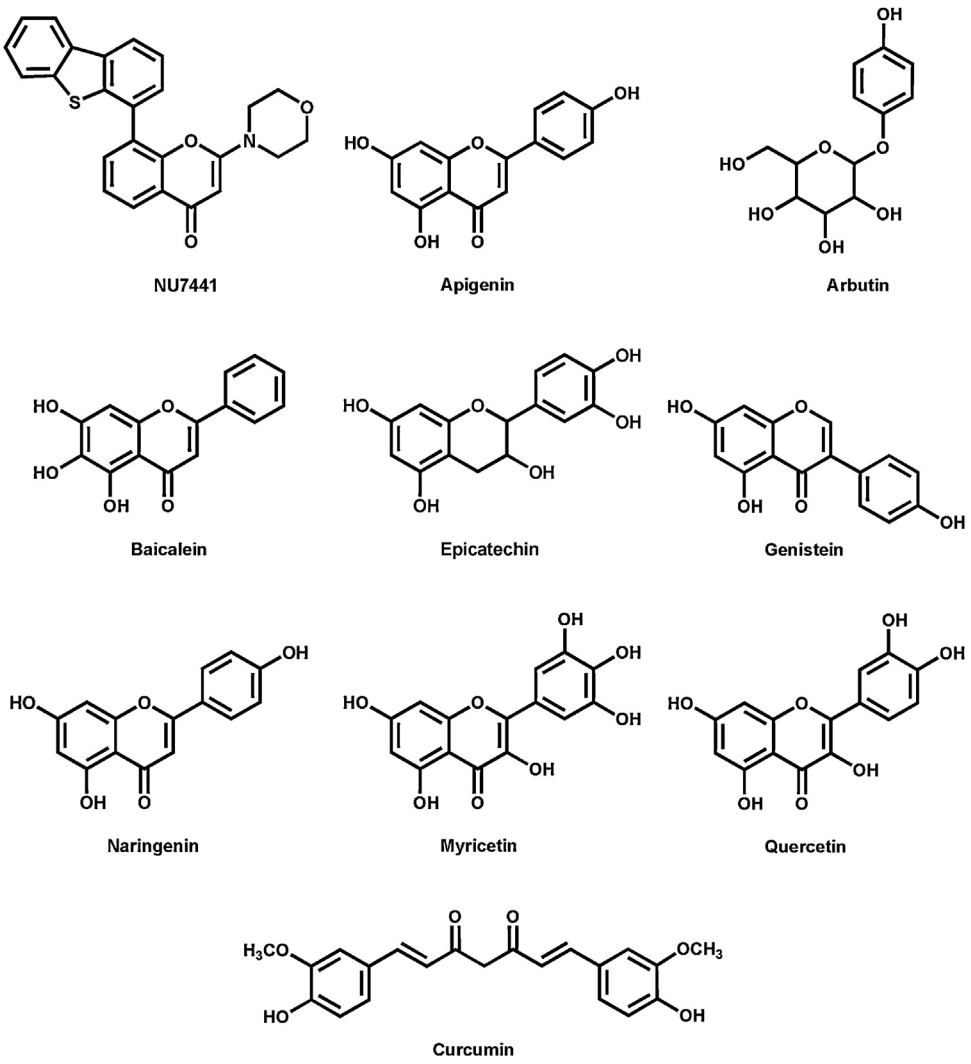

Fig. 1. Structures of the investigated natural compounds. Nine flavonoids from seven different classes, arbutin, curcumin and indole-3-carbinol were investigated in this study.

Table 2
Classification, main sources and non-lethal concentrations of investigated natural compounds.

Group	Class	Name	Main sources	Concentration applied for NHEJ experiments[a]
Flavonoids	Flavone	Apigenin	Parsley, plantain	10 μM[b]
		Baicalein	Baikal Skullcap	30 μM[c]
	Flavanone	Naringenin	Grapefruit	30 μM[c]
		Sakuranetin	Blackcurrant	30 μM[c]
	Flavonol	Myricetin	Grape, berries	30 μM[c]
		Quercetin	Green tea, red onions	30 μM[c]
	Flavanol	Epicatechin	Green tea, cocoa bean	30 μM[c]
	Chalcone	Chalcone	Liquorice	30 μM[c]
	Isoflavone	Genistein	Soybean, chickpea	30 μM[c]
Polyphenols	Hydroquinone	Arbutin	Bearberry	30 μM[c]
	Curcuminoid	Curcumin	Curcuma	5 μM[b]
Glucosinolates	Glucosinolate-derivative	Indole-3-carbinol	Cruciferous vegetables	30 μM[c]

[a] As determined by MTT test (24 h; tested concentration range, 0.8–200 μM).
[b] IC_{10}.
[c] Non-lethal concentration (IC_{10} > 30 μM).

2.8. Statistical analysis

Nuclear proteins were extracted in duplicates. For each experiment, one control nuclear extract and two natural product-pretreated nuclear extracts were simultaneously prepared; four NHEJ assays were performed for each nuclear extract, and each assay was analyzed by on-chip electrophoresis in du- or triplicates (one replicate per chip). For each run, data were expressed as percentages of monomer area among all the detected band areas (monomer + multimers up to tetramers). Raw data were used for statistical analyses; for graphical representations, data were expressed as proportions of monomers relative to the corresponding control experiments.

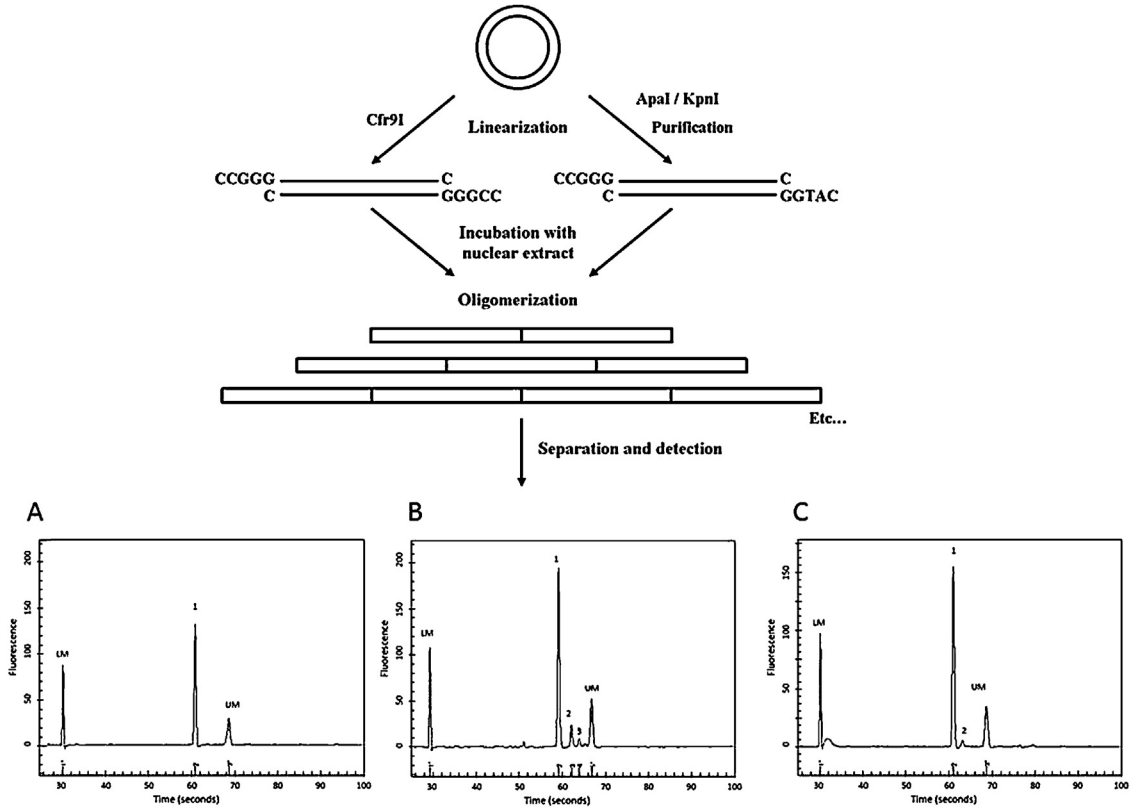

Fig. 2. Protocol for *in vitro* NHEJ assay (small intestinal epithelial cell line, FHs 74 Int.). (A) Negative control (no proteins); (B) Control (proteins of untreated cells; % multimers set at 100%); (C) NU7441 (10 μM; 20 min nuclear extract pre-treatment on ice followed by end-joining reaction; % multimers, 18%). Peaks: (1) plasmid monomer; (2) dimer; (3) trimer; (LM) lower molecular weight marker; (UM) upper molecular weight marker.

Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) using one-way ANOVA with *post hoc* Student's *t* tests (Bonferroni correction); p values < 0.05 were considered significant.

3. Results

3.1. The cell model

To avoid using cancer cell lines with uncertain or unknown DNA damage signaling and DNA repair status [32], we selected a human small intestinal cell line. These cells, normal and non-transformed, are expected to have conserved DNA repair competencies. Derived from normal human fetal intestine, FHs 74 Int cells have been reported to show mature epithelial-like characteristics [65]. The small intestine constitutes an organ where the vast majority of food digestion and absorption take place, and this cell line has already been used in many studies concerning food effects on biological processes [66–68].

3.2. Protocol using plasmid template with cohesive ends

A protocol based on cohesive ends plasmids was applied to the nuclear extracts obtained from cells treated with each of the selected twelve natural compounds. Apigenin, baicalein, and curcumin produced a significant inhibitory effect on plasmid oligomerization while arbutin, chalcone, epicathechin, myricetin, and sakuranetin exerted a significant stimulation. Treatment of cells with genistein, naringenin and quercetin showed no significant effect ($p > 0.05$) (Fig. 3). The DNA-PKcs inhibitor NU7441 caused a significant ($p < 0.01$) inhibition of plasmid oligomerization of $82 \pm 12\%$ ($n = 3$) compared to the control.

3.3. Protocol using plasmid template with incompatible ends

The nuclear extracts obtained with cells treated by the two most potent inhibitors (apigenin and curcumin), stimulators (myricetin and sakuranetin) and one compound without significant effect (quercetin) were then tested with linearized plasmids bearing incompatible ends. Indeed, the NHEJ oligomerization of plasmid substrates with complementary ends may strongly depend on the efficiency of the ligation step, while substrates with non-complementary ends allow a more thorough probing of the entire NHEJ process as the ends must first be converted into a ligatable form by filling DNA synthesis and/or bases trimming [5].

The two most potent positive modulators of oligomerization, myricetin and sakuranetin, also significantly stimulated the oligomerization of incompatible-ends plasmids (Fig. 4). The inhibitory effect of curcumin and the indifferent effect of quercetin were also consistent in this second model; by contrast, treatment with the negative modulator apigenin had no significant effect on the oligomerization of plasmids with incompatible ends (Fig. 4).

4. Discussion

The automated on-chip microelectrophoresis validated protocol which we developed [29] was applied for the present study without particular problems, consistently yielding reproducible data. The inhibition (82%) of oligomerization by the DNA-PKcs inhibitor NU7441 indicates that the assay effectively measures NHEJ. Working with a normal cell model implies slowly growing cells and limited number of passages and so limits the number of tests possible. To ensure confidence in the generated data and to allow screening-type studies, the analytical method was previously validated by investigating both reproducibility and ruggedness [29].

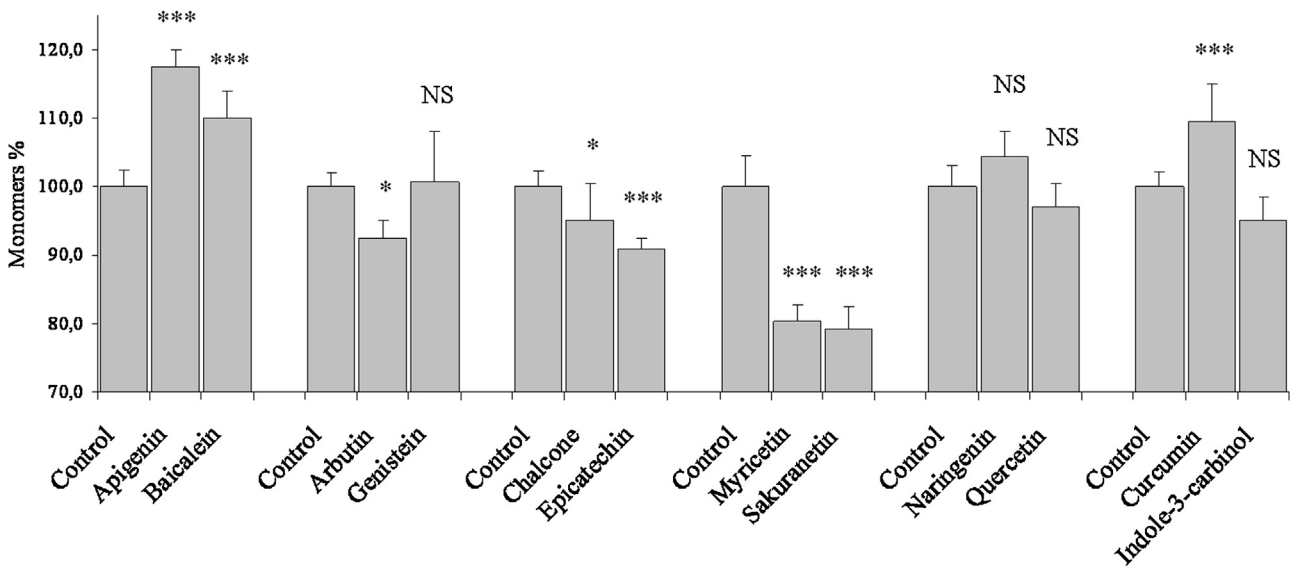

Fig. 3. Influence of natural products on the protocol with cohesive ends plasmid substrates. Effects of the twelve investigated natural compounds on plasmid oligomerization measured by microchip electrophoresis; the proportions of monomers are presented (reaction temperature, 37 °C; reaction time, 20 min; four biological replicates analyzed in du- or triplicate; mean + S.D. are expressed and data were normalized to their respective control). An increase in monomer proportion indicates a decrease in NHEJ efficacy; conversely a decrease in monomer proportion indicates activation of NHEJ. One-way ANOVA with *post hoc* Student's *t* tests (Bonferroni correction) for the difference between the effect of natural products and their respective controls (NS, $p > 0.05$; $^{*}p < 0.05$; $^{***}p < 0.001$).

The application of this method to an intestinal non-cancerous cell model indicates that some natural polyphenols affect the NHEJ DNA repair capacity.

4.1. Consequences of NHEJ modulation

The likely consequences of positive or negative modulation of NHEJ may be difficult to predict. Defects in NHEJ or HR activities clearly cause genome instability and promote tumorigenesis [4]. NHEJ and HR are often described as "error-prone" and "error-free" pathways, respectively. However, this is an oversimplification; both NHEJ and HR can be precise or not, depending on a series of factors [4,69]. In mammalian cells, 25–50% of DSBs produced by nucleases are repaired by precise NHEJ; and when ends cannot be precisely rejoined, NHEJ uses microhomologies to repair, leading to small deletions and/or insertions. HR uses homologous sequences elsewhere in the genome (sister chromatids, homologous chromosomes, or repeated regions on the same or different chromosomes) to repair a DSB. This repair can be completely accurate if the template is perfectly homologous (in the case of sister chromatids), but repair polymerases are more imprecise than replicative polymerases, and point mutations are frequent at DSB repair sites. When the template is not totally homologous, HR can result in localized loss of

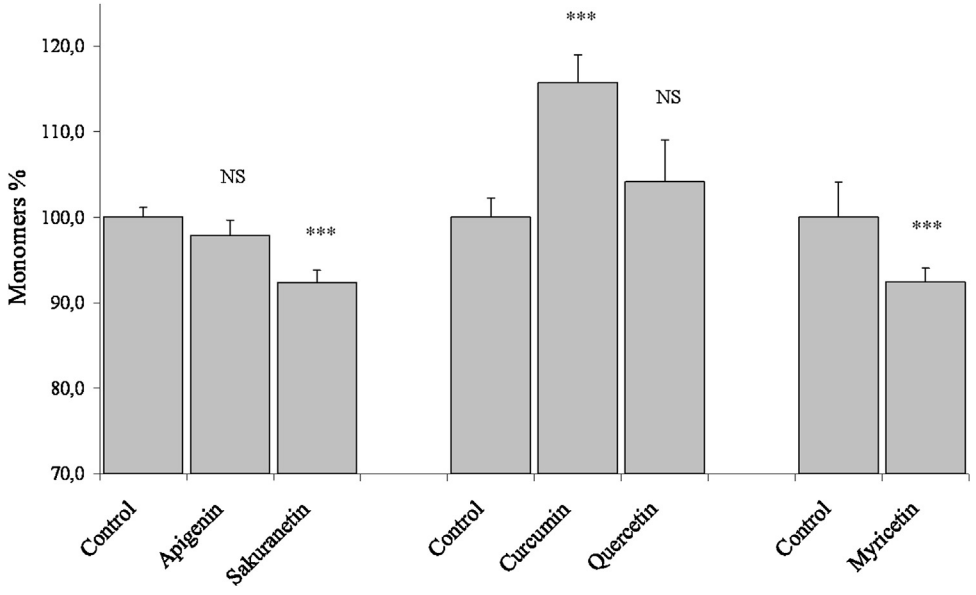

Fig. 4. Influence of selected natural products on the protocol with incompatible-ends plasmid substrates. Effects of the five selected natural compounds on plasmid oligomerization measured by microchip electrophoresis; the proportions of monomers are presented (reaction temperature, 25 °C; reaction time, 60 min; four biological replicates analyzed in du- or triplicate; mean + S.D. are expressed and data were normalized to their respective control). An increase in monomer proportion indicates a decrease in NHEJ efficacy; conversely a decrease in monomer proportion indicates activation of NHEJ. One-way ANOVA with *post hoc* Student's *t* tests (Bonferroni correction) for the difference between the effect of natural products and their respective controls (NS, $p > 0.05$; $^{***}p < 0.001$).

heterozygosity and potentially reciprocal translocations, if associated with crossovers [4,70].

The main factors implicated in the classical NHEJ (DNA-PK complex, XRCC4-DNA Ligase IV, Artemis) have been shown to protect the mammalian genome [71]; indeed a disruption in NHEJ implies higher rates of genome rearrangements and acute sensitivity to DNA damaging agents, including ionizing radiation [70,72].

Cells seem to accumulate small mutations resulting from NHEJ, rather than chromosomal translocation or deletions caused by HR if the choice of the homology partner is inappropriate [73]; this indicates the superior importance of maintaining chromatin structural integrity over that of sequence fidelity [9]. Activation of NHEJ then appears to participate effectively in the stability of the genome, even at the cost of point mutations that may need other repair pathways for correction. Some common dietary compounds modulate NHEJ and may thus influence the outcome of a genotoxic insult. Preliminary data of the present study point to the need for further mechanistic and *in vivo* studies.

4.2. Tested polyphenols and chemoprevention

A series of reports indicates that polyphenols tested in the present study can prevent the appearance and development of carcinoma [74–78]. Notably, modifications observed in the expression of a series of genes in flavonoid-treated cells suggest an effective role in genomic stability [79]. Cancer chemoprevention related activities of tested polyphenols are summarized in Table 1 Given the limitations of our *in vitro* tests system, further mechanistic work is clearly needed to conclude that the molecules positive in the present study effectively involve NHEJ modulation, notably by examining (i) the capacity for end repair in cell extracts that are immunodepleted for known NHEJ factors, including DNA-PKcs, Ku proteins, Xrcc4; and (ii) the influence of modulators on the sequence of new junctions. Apigenin already appeared as a modulator of strand break rejoining in our comet assay-based investigations [32] and is certainly worthy of further investigation.

In a previous paper [29], we reported that quercetin markedly stimulates NHEJ on an immortalized LHCN-M2 human myoblast cell line. By contrast, the present experiments on normal intestinal epithelial cells indicate no effect of quercetin on plasmid oligomerization. This could be explained by several factors: there could be different responses to polyphenols according to cell types [80,81]; the LHCN-M2 human myoblast cell line was immortalized by transduction with human telomerase reverse transcriptase (hTERT) and cyclin-dependent kinase 4; the NHEJ efficiency seems to differ according to the telomerase status, as shown in comparing immortalized and senescent cells [82]; the complex connections telomerase-factors of NHEJ [83] and telomerase-quercetin [84,85] may give rise to unexpected interactions; and quercetin and other flavonoids are known to be conjugated to form glucuronides and/or sulfates by enzymatic activities found in intestinal epithelial cells, before they reach the liver and the circulation [86]; conjugation of quercetin may decrease its NHEJ modulating activity.

These conflicting data show the importance of using a cellular model as close as possible to real *in vivo* situations. The normal epithelial cells indeed appear as a more suited model.

4.3. Redox properties of tested polyphenols

Polyphenols, well-known for antioxidant properties, can also be pro-oxidant, depending on many factors in their near-environment; although their exact cellular activity (anti- or pro-oxidant) is debated [87], their redox properties have been previously correlated with cancer chemoprevention, with induction of a cancer-protective enzyme, NAD(P)H-quinone oxidoreductase 1, with topoisomerase inhibition and with inflammation suppressive capacity [88]. Such redox properties may be a clue to explain the observed NHEJ modulations. On the one hand, direct redox modification of some repair factors may modulate NHEJ. On the other hand, redox signaling and oxidation of peptides and proteins are important in DNA damage signaling; as our assay measures the NHEJ capacity (treatment of cells for 24 h and extraction of total nuclear proteins), it takes into account any response to the potential modulators that would for example lead to nuclear translocation and/or transcription. Bensasson et al. [88] have measured and/or computed the energy of the highest occupied molecular orbital

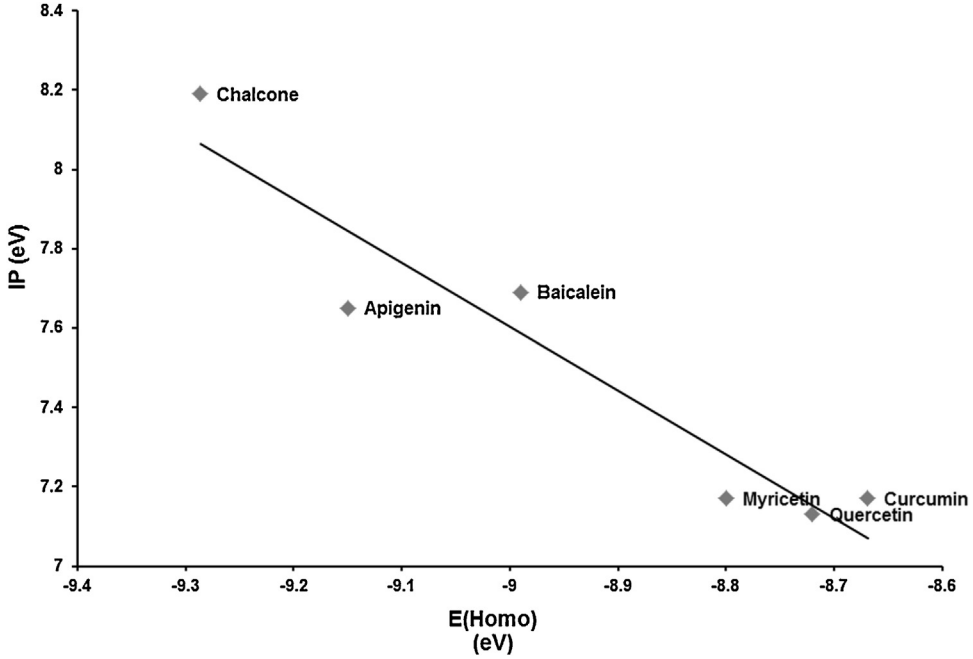

Fig. 5. Redox properties of investigated flavonoids. Energy of the highest occupied molecular orbital (*E*(homo)) and the ionization potential [IP or E($M^{\bullet+}$/M)]. Graph plotted from the data of Bensasson et al. [88].

(*E*(homo)) and the ionization potential [IP or E($M^{\bullet+}$/M)] for many polyphenols, including some of the flavonoids tested here; we plotted the data from Bensasson to show, in the right inferior quadrant, the best antioxidants (Fig. 5). In this limited dataset, however, no correlation is observed with NHEJ modulations reported in Figs. 3 and 4.

5. Conclusion: modulation of NHEJ by natural compounds

Most of the chemoprevention studies performed so far (exemplified in the previous section) investigated modulatory effects on cell cycle, death mechanisms, inflammation and hormone-mediated intracellular signaling and global effects on DNA damage excision. As no compounds are known to modulate the NHEJ pathway, it would be interesting to know which types of molecular structures can interfere, either positively or negatively. These might lead to a better understanding of cancer avoidance mechanisms, to nutritional advice for cancer chemoprevention and to the development of nutraceuticals with original health claims. Inhibitors of DNA repair may be candidates for new drug adjuvants to current cancer treatment modalities. However, preventive or therapeutic applications need to consider the *in vivo* bioavailability and biotransformation of natural products. Further studies are clearly needed to clarify their possible role in human cancer chemoprevention by interference with DNA repair pathways.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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