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DNA Damage and Repair Mechanisms Triggered by Exposure to Bioflavonoids and Natural Compounds

Donna Goodenow, Kiran Lalwani and Christine Richardson

Abstract

Eukaryotic cells use homologous recombination (HR), classical end-joining (C-NHEJ), and alternative end-joining (Alt-EJ) to repair DNA double-strand breaks (DSBs). Repair pathway choice is controlled by the activation and activity of pathways specific proteins in eukaryotes. Activity may be regulated by cell cycle stage, tissue type, and differentiation status. Bioflavonoids and other environmental agents such as pesticides have been shown to biochemically act as inhibitors of topoisomerase II (Top2). In cells, bioflavonoids directly lead to DNA double-strand breaks through both Top2-dependent and independent mechanisms, as well as induce DNA damage response (DDR) signaling, and promote alternative end-joining and chromosome alterations. This chapter will present differences in expression and activity of proteins in major DNA repair pathways, findings of Top2 inhibition by bioflavonoids and cellular response, discuss how these compounds trigger alternative end-joining, and conclude with implications for genome instability and human disease.

Keywords: environmental compounds, bioflavonoids, DNA double-strand breaks, topoisomerase II, DNA break repair, genome instability

1. Introduction

The faithful repair of deoxyribonucleic acid (DNA) lesions is central to the maintenance of genomic integrity [1]. DNA double-strand breaks (DSBs) occur during normal developmental processes including meiosis, mating-type switching, V(D)J recombination, antigen receptor gene rearrangement, and also through normal activity of topoisomerase II (Top2) [2–5]. DSBs also result from exposure to exogenous sources such as ionizing radiation (IR), reactive oxygen species, and chemotherapeutic agents including inhibitors of Top2 [6–9]. Aberrant repair of DSBs may be mutagenic and result in cell lethality or promote oncogenic transformation. Repair of DSBs in eukaryotes occurs by either homology-dependent or homology-independent (also known as end-joining or illegitimate) mechanisms [10–13]. In yeast, homology-dependent repair predominates over end-joining [10, 14]. In mammalian cells, direct examination of repair products has demonstrated the

predominant use of end-joining [13]. The majority of studies generate targeted DSBs by endonucleases or lasers, and introduce artificial repair substrates into the system [15]. However, exposure to natural compounds can lead to multiple DSBs in a variety of chromatin regions and contexts [16–20]. Understanding how cells respond to these compounds and repair damage caused by them has important implications for genome stability.

Bioflavonoids are natural compounds in soy, fruits, vegetables, tea, coffee, and wine, and contained in energy drinks and dietary supplements [21–24]. Bioflavonoids are also in pesticides and flame retardants [25–27]. Bioflavonoids inhibit the enzyme topoisomerase II (Top2) to promote DSBs, and recent studies have elucidated the cellular mechanisms used to repair the DSBs induced by bioflavonoids [16, 28, 29]. This chapter will discuss cell type differences in expression and activity of proteins in major DNA repair pathways, summarize findings of cellular response to bioflavonoids and Top2 inhibition, discuss how these compounds trigger alternative end-joining, and conclude with implications for genome instability and human disease.

1.1 DNA double-strand break repair

There are three main repair pathways to deal with DNA double-strand breaks (DSBs) in eukaryotic cells. These include classic nonhomologous end-joining (C-NHEJ) (**Figure 1A**) that modifies and allows for ligation of ends, alternative end-joining (Alt-EJ) that generates short overhangs or exposes small regions of homology via resection to promote ligation of ends (**Figure 1B**), and homologous recombination (HR) that uses a homologous sister chromatid, chromosome, or other sequence as a template to direct repair synthesis (**Figure 1C**) [10, 30]. HR is the most accurate using a homologous template as a donor sequence. DSBs are recombination initiators in both meiotic and mitotic cells [31–33]. However, HR has the most protein involvement, is tightly regulated, largely limited to S phase, and kinetically slow. C-NHEJ is utilized throughout the cell cycle and is kinetically fast. Alt-EJ is less well characterized than the other two and considered a backup repair mechanism when HR or C-NHEJ cannot be used. For a DSB to be repaired by HR or either of the end-joining pathways, damage must first be sensed, then signal transduction pathways must be activated for the DNA damage response (DDR) to bring proteins necessary for repair to the site(s) of damage. Indirect signaling and direct repair protein levels along with histone modifications appear to direct DSB repair pathway selection [34–37]. Despite decades of extensive study of DSB repair, scientists continue to identify and characterize new factors mechanistically involved in DSB end processing, repair itself, as well as pathway choice [38].

1.2 End-joining pathway choice

Repair of DNA DSBs by C-NHEJ or Alt-EJ is characterized by ligation of two DSB ends in close proximity to each other (**Figure 1A** and **B**). Initial binding of the Ku70–80 heterodimer competes with poly(ADP-ribose) polymerase 1 (PARP1) for binding to the DSB. If Ku70–80 binds first there is minimal end processing and C-NHEJ is used [39–44]. For C-NHEJ, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited to the Ku complex. DNA-PKcs can determine if the ends are blunt, as from a nuclease cleavage or from RAG during V(D)J recombination, or if there are overhangs or protein/group adducts. If the break is clean, DNA-PKcs recruits XRCC4-XLF and LigaseIV, and these proteins work together to ligate the DNA ends [39, 42, 45]. However, if there is an overhang or proteins are attached to the break site, DNA-PKcs recruits the ARTEMIS complex for processing.
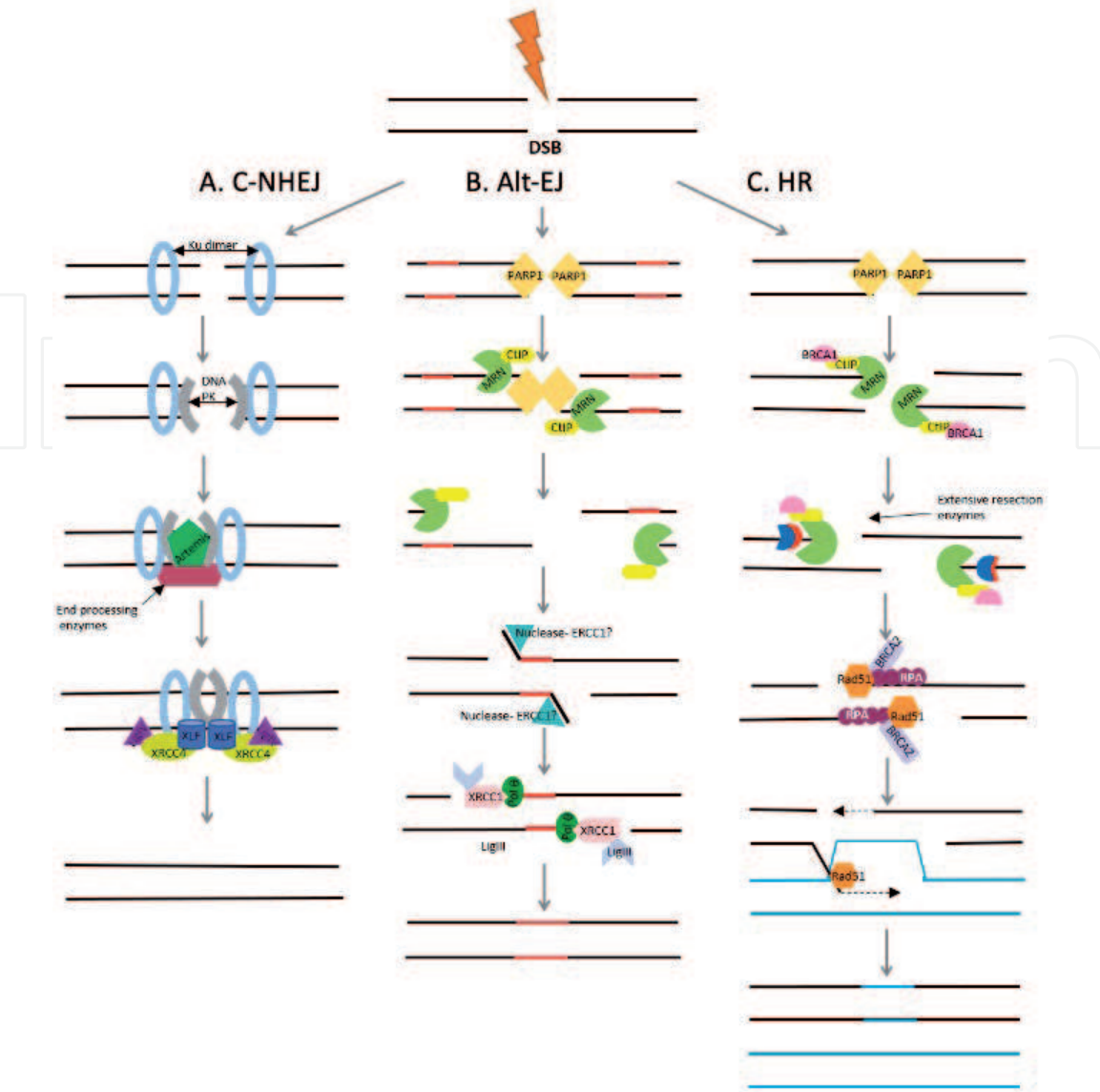
Figure 1.
The DNA double-strand breaks (DSB) are repaired by the three pathways; these are – A) non-homologous end joining (C-NHEJ) which modifies the ends and allow ligation of the broken ends to repair the DSB; B) alternate end-joining (alt-EJ of EJ) creates short DNA overhangs with small regions of homology and ligates the resected broken ends; and C) homologous recombination (HR) that uses a homologous sequence from sister chromatid or homologous chromosome or a homologous sequence within the genome.

ARTEMIS can release protein groups and with its nuclease activity to digest the DSB ends until they are blunt to facilitate ligation of the ends [46].

If PARP1 binds to the DSB before Ku70–80, it immediately adds branched poly(ADP-ribose) (PAR) groups to itself and histones in close proximity. The branched PAR recruit the Mre11-Rad50-Nbs1 (MRN) complex to process the ends and proceed by HR or Alt-EJ. Alt-EJ seems to act as a salvage repair mechanism for when HR and C-NHEJ are blocked. It is likely that Alt-EJ occurs when processing for HR has started following PARP1 binding to the break first, Ku70–80 is depleted, because the DSB ends have proteins bound to block template invasion, or the cell is in G1 phase of the cell cycle no homologous template is readily available for repair. Alt-EJ involves MRN and CtIP to resect the DSB ends in a 3′ to 5′ fashion, termed short range end resection, of 5–25 nucleotides to create short DNA overhangs with small regions of homology. Polymerase θ is utilized in Alt-EJ. After processing, XRCC1 and LigaseIII act in a complex to ligate the ends and remove the overhanging bases. Alt-EJ is more mutagenic than HR or C-NHEJ and associated with chromosomal rearrangements and translocations [44, 47–49].

1.3 Homologous recombination requires chromatin remodeling and DDR

To initiate HR (**Figure 1C**), PARP1 is recruited to the DSB first and immediately adds branched PAR groups to itself and histones in close proximity. The branched PAR recruit the MRN complex and inactive ATM kinase dimers with the acetyl-transferase TIP60 attached. PARG quickly removes the PAR groups allowing the MRN complex to bind to the DSB. MRN allows ATM to bind at the DSB and activate through auto-phosphorylation and acetylation by TIP60, thereby allowing TIP60 to dissociate. Once active, ATM will phosphorylate a large number of target proteins including the MRN complex and CtIP that process DSB ends [12, 34, 42, 50].

Chromatin remodeling is extensive and required for HR-mediated DSB repair. Histone H2AX is phosphorylated by ATM as well as acetylated by TIP60. Phospho-H2AX (γ-H2AX) has some chromatin remodeling functions and acts as a signal to recruit additional proteins involved. γ-H2AX will spread away from the DSB to decorate chromatin up to 2 Mb away. MDC1, which assists with chromatin remodeling, becomes phosphorylated by ATM and recruits RNF6 dimers that have ubiquitination functions. HERC2 associates with phosphorylated RNF6 and appears to recruit PIAS4 which has SUMOlyation capabilities. RNF6 becomes SUMOlyated and mono-ubiquitinates histones in the area, which recruits RNF168, another ubiquitin ligase, that is SUMOlyated and poly-ubiquitinates nearby histones. The poly-ubiquitin trees tether BRCA1-A complexes by RAP80 mediators. These complexes cause histone modifications that bring in 53BP1, which has more histone remodeling functions and can inhibit MRN and CtIP-mediated end resection [34, 39, 43, 50].

Phosphorylation of target proteins by ATM also triggers DDR. Chk2 has protein kinase activity allowing it to phosphorylate a number of effector proteins in the cell cycle checkpoint including p53 which can be modified by either Chk2 or ATM (or ATR or Chk1). ARF protein (p14) seems to stabilize TIP60 interactions with ATM for better activation and is associated with maintaining genome stability [34].

While the histone remodeling is occurring and other proteins are being recruited, MRN and CtIP resect the DSB ends short range end resection, then Exo1 or Dna2 nucleases act in long range end bidirectional resection in a 5′ to 3′ direction away from the DSB. Exo1 has dsDNA nuclease function, while Dna2 must act with a helicase like BLM or WRN to unwind DNA for its ssDNA nuclease abilities [34, 37, 43, 51]. While long range end resection is occurring, RPA binds to the 3′ ssDNA overhang to protect from nucleases. After this resection, one type of HR can occur called single strand annealing (SSA), where the two pieces of RPA coated DNA associate with one another with the help of Rad52 and if regions of homology are found they anneal to one another. Non-homologous flaps are cleaved off by enzymes like XPF-ERCC1 and ligated by LigaseIII. This type of HR can cause large deletions [12, 43, 50, 52].

Canonical HR, as well as break-induced replication (BIR) and synthesis-dependent strand annealing (SDSA) use BRCA 1 and 2 with Rad51 for homology searches that cause strand-invasion, D-loop formation and resolution/dissolution. RPA must be dissociated from the ssDNA for Rad51 binding, mediated by DSS1 and BRCA2 which displace RPA and stabilize ATP on Rad51 increasing its binding affinity for the ssDNA. Once Rad51 is loaded on the DNA and the nucleofilament has formed, it can invade neighboring DNA to search for homology with BRCA1 [34, 43, 50]. Homology less than 7 nt in length is a weak interaction and Rad51 not sufficient to initiate HR, but 7 nt or longer allows the strand to interact more strongly [50]. If significant homology is present, the ATP on Rad51 is hydrolyzed causing the dsDNA to dissociate and the nucleofilament anneals with the template strand.

RPA stabilizes this D-loop formation by binding to the displaced strand. DNA Polymerase δ or ε uses the invading strand as a primer to initiate synthesis [12, 39, 50, 53]. Resolution can happen with crossover or non-crossover products and different sets of resolvases mediate this process. For one-sided ends that utilize BIR, DNA Pol δ is used and synthesis continues until the end of the chromosome causing gene conversion that can be highly mutagenic [54].

2. Topoisomerase II, inhibitors and poisons

Topisomerase II (Top2) is a regulatory enzyme that relaxes supercoiled DNA for transcription (Top2β) and replication (Top2α). As shown in **Figure 2**, Top2 acts in a multistep cleavage and religation reaction: (1) Top2 binds to two dsDNA molecules at Top2 recognition sequences; (2) a transient DSB is generated in the first DNA helix (G-segment) creating a cleavage complex; (3) ATP hydrolysis drives a conformational change allowing the second dsDNA helix to pass through the DSB; (4) Top2 mediates religation of the DSB and the T DNA segment is released; (5) the G DNA segment is released and the enzyme returns to its original conformation (**Figure 2**). A catalytic Top2 inhibitor such as dexrazoxane acts to prevent DNA from binding to Top2 at step 1 preventing any part of the catalytic cycle [55–59].
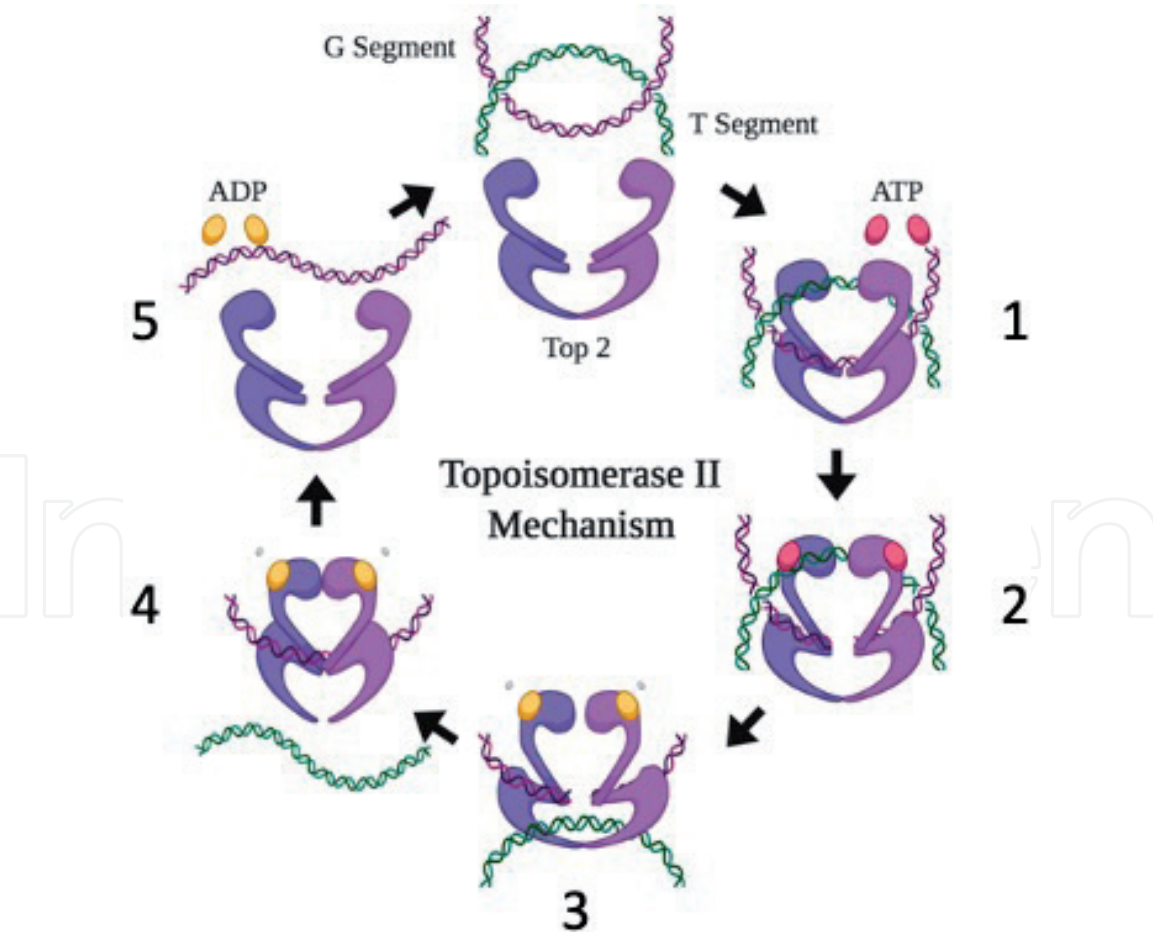

Figure 2.
Top2 acts in a multistep cleavage and religation reaction. 1) Top2 binds the G and T dsDNA molecules at Top2 recognition sequences. 2) ATP binding catalyzes the DNA DSB in the G segment, which allows the T segment to pass through the break. (3) ATP hydrolysis drives a conformational change allowing the second dsDNA helix to pass through the DSB; (4) Top2 mediates religation of the DSB and the T DNA segment is released; (5) the G DNA segment is released and the enzyme returns to its original conformation.

3. Bioflavonoids and other natural compounds as Top2 inhibitors

A class of chemical compounds called bioflavonoids are contained in soy, fruits, vegetables, tea, coffee, wine, energy drinks, and dietary supplements [21–27]. Bioflavonoids are characterized by multiple phenolic rings that are central to their ability to inhibit the enzyme Top2 in a similar manner to the chemotherapeutic drug etoposide [16, 28, 29]. Some pesticides and flame retardants also contain multiple phenolic rings and have been identified as Top2 inhibitors. Bioflavonoids are separated into 12 different sub-classes based upon their structure; however only six are contained in dietary sources: flavanols, flavonols, flavones, isoflavones, flavanones, and anthocyanidins (**Figure 3**) [60, 61].

3.1 Isoflavones

Isoflavones are polyphenolic secondary plant metabolites produced through the flavonoid-producing phenyl-propanoid synthesis pathway (**Figure 4**). In order for isoflavone production, the plant must express the isoflavone synthase enzyme which converts flavanone precursors into isoflavones. This isoflavone synthase is only expressed in legumes and a few other select species. Plants with the highest concentrations of isoflavones are soy, red clover, and kudzu. The amount of isoflavone depends upon the conditions the plants were grown, and the final concentration of isoflavones in food products (including dietary supplements) depends upon which portion of the plant is used and the processing methods. Genistein, daidzein, glycitein, formononetin, biochanin A and irilone are the main isoflavones isolated
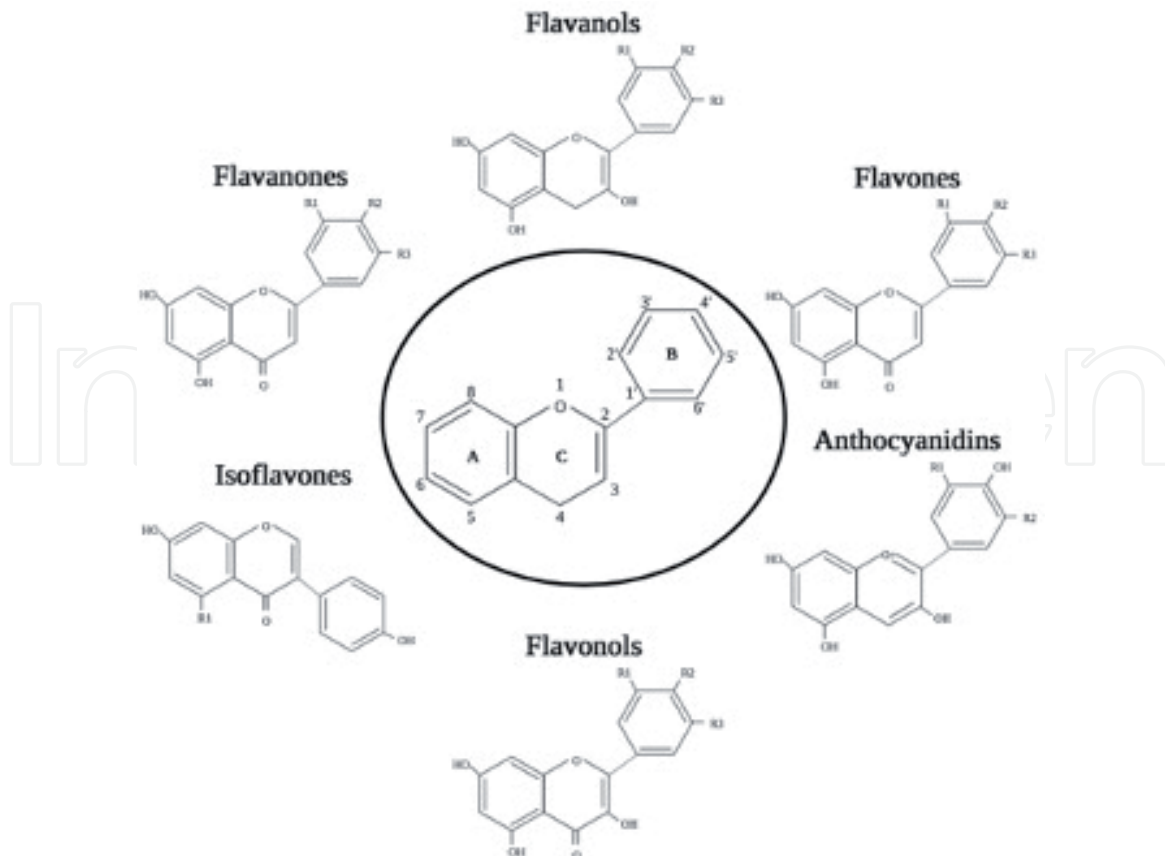

Figure 3.
Basic chemical structures of dietary bioflavonoids. The middle circled backbone represents the general bioflavonoid poly-phenol ring structure. The six structures surrounding show the general structural differences between the sub-groups.
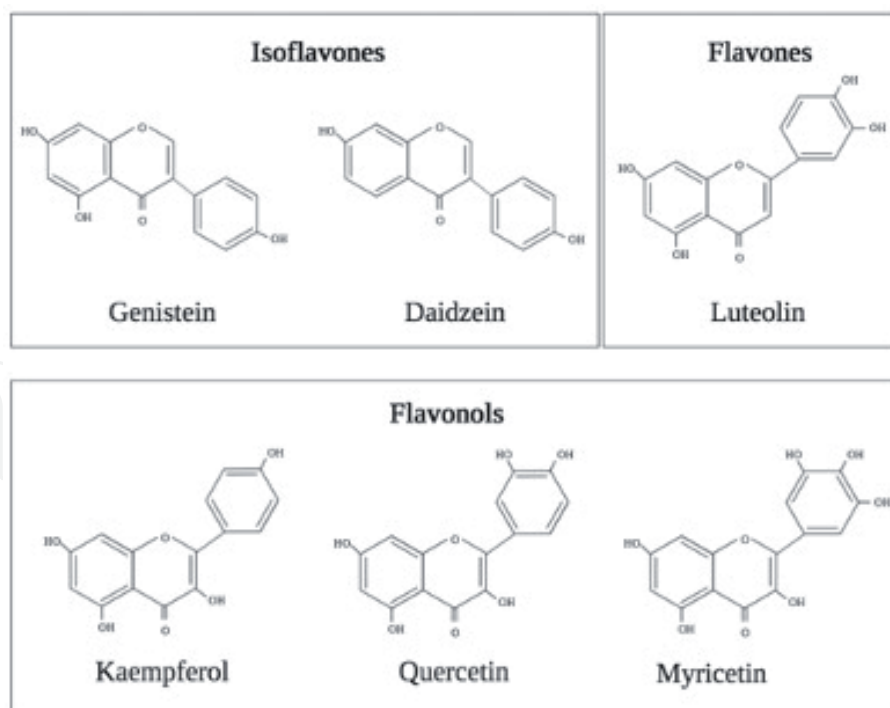
Figure 4.
Structure of commonly found bioflavonoids flavanols: Genistein and Daidzein, flavonols: Kaemferol quercetin and Myricetin, and flavones: Luteolin.

from plants [60, 62, 63]. Genistein and daidzein are of particular interest due to their high concentration in soy products [60]. Genistein is an estrogen derivative available at health food stores as dietary and menopausal supplements, and a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas [23, 64, 65].

Interest in isoflavones has spiked in the past 20 years. This is due to the attribution of consumption of isoflavone-containing products with lower occurrences of coronary heart disease, breast and prostate cancer. This hypothesis derived from observations that citizens of Asian countries have lower incidence of these diseases compared to citizens of Western countries, and that citizens in Asian countries typically ingest 8-50 mg/day of isoflavones compared to citizens in Western countries who ingest only 0.1–3.3 mg/day [66, 67] .

Due to this potential health relevance, studies examined the impact of high intake of isoflavones, but the results have been inconclusive [62]. In animal models, increased genistein intake resulted in increased rates of pituitary and mammary gland tumors and stimulated MCF-7 tumor growth. Additionally, while increased genistein intake in post-menopausal women in Asian countries decreased breast cancer risk, this decreased risk was not sustained in post-menopausal women in Western countries, including both native inhabitants and Asian immigrants. Some studies, particularly of British women, showed that increased serum genistein levels in women with early stage breast cancer had increased transcription of cell cycle progression and cell proliferation genes [62].

3.2 Flavones

Flavones are the end product of a complex multi-step synthetic pathway that occurs within a wide variety of plants (**Figure 4**). This pathway begins with phenylalanine that is converted through the generalized phenylpropanoid pathway that synthesizes most flavonoids. Subsequently, p-coumaroyl-CoA must be synthesized into chalcone with chalcone synthase. Chalcone can be isomerized into a flavanone

by chalcone isomerase. Finally, flavone synthase class I or II enzymes catalyze the synthesis of a flavone from flavanones. Flavones, similar to flavonols, can protect the plant from UV-B radiation. Flavones have the additional ability to provide protection against biological attacks from pathogenic microbes by acting as signaling molecules to activate differential gene transcription to prevent the growth of microorganisms after invasion. Additionally, flavones can be expressed to deter insects and nematodes from eating the plant or to interfere with the growth and reproduction of other plants [60].

Flavones are found across a variety of plant species, and expression of flavones appears to be widespread within the plant, from the roots to the leaves. However, though flavones are found throughout the plant kingdom, they are found much less commonly in fruits and vegetables as compared to flavonols. Apigenin and luteolin are the main flavonols contained in food sources including celery, parsley, thyme, red peppers, and fruit skins [61, 68]. In humans, flavones, much like isoflavones and flavonols, seem to have antioxidant and anti-tumor capabilities and to affect signal transduction pathways [69].

3.3 Flavonols

Flavonols are primarily in fruits, vegetables, red wine, and tea and they compose the largest portion of humans' bioflavonoid intake given their distribution across a wide number of plant species (**Figure 4**) [61]. Within plants it has been shown that flavonols have the ability to protect the plant against UV-B damage, and they protect the plants against oxidative damage with their antioxidant capability [70, 71]. Scientists and physicians want to determine ways to utilize the antioxidant capability of flavonols in human populations as a protectant against cardiovascular and neurological disease and against exercise induced oxidation in smokers and athletes [72, 73].

The most common flavonols in foods are quercetin, kaempferol, myricetin, and fiestin, with a majority of published literature focusing upon the first three. Similar to isoflavones the concentration of flavonol in the food product depends upon the plant, the growth conditions, and the part of the plant used. Flavonols are found in highest concentrations in the leaves, flowers, and fruits, which are exposed to sunlight; the exception to this being onions which grow below ground [70, 71]. The human dietary source of flavonols is dependent on culture and region. Humans residing in Asian countries typically ingest flavonols through green tea, while the Netherlands, United States and Denmark inhabitants mainly ingest them from onions, apples, and tea. Citizens of Mediterranean areas ingest flavonols from green vegetables. Within Italy, red wine is the main source of flavonols, though inhabitants of Northern villages also have a high intake from salads, soups, fruits. The prevalence of flavonols in the human diet has produced a large interest in understanding their multiple cellular effects and potential impact on human health [70].

3.4 Additional compounds as Top2 inhibitors

Additional natural compounds other than bioflavonoids may also act as inhibitors of Top2. Bakuchicin from the furanocoumarin family is present in fruits and legumes [74]. In research conducted to study DNA-polymerase inhibition activity of *Psoralea corylifolia L.* (Leguminosae), bakuchincin was found to be a weak Top2 inhibitor [75]. Additional reported naturally occurring Top1 and Top2 inhibitors are benzophenone compounds such as xanthochymol and Garcinol at effective concentrations comparable to those of etoposide ($\sim$25 – 100 μM) [76, 77]. A comparative study between the naturally occurring constituent of black seed thymoquinone

used as a spice in eastern cooking and a known Top2 inhibitor 1,4-benzoquinone showed structural and functional similarity between the two compounds and the ability to induce DNA cleavage [78].

Triterpenoids are present in plants, widely distributed within the root, stem, leaves, bark. They are components in the waxy covering of fruits and herbs such as jujube, lavender, and thyme [79]. Triterpenoids have two major components, C5 units and isopentyl diphosphate [80], and are generally present as saponins that act as defense chemicals for protection against microbes. Triterpenoids betulin lupane and oleanane from the bark of *Phyllanthus flexuosus*, derivatives of betulinc acid, and oxygenated derivatives of oleanane called celastroloids were reported to act as human Top2 inhibitors to varying degrees [81–84]. In addition, betulinic acid which is an oxidative derivative of betullin inhibits cell proliferation by inhibiting topoisomerase-DNA binding and suppressing NF-κB activation [83].

Halogenated compounds in household and baby products include polychlorinated biphenyls (PCBs), detectable in indoor carpets, and polybrominated diphenyl ethers (PBDEs), used as flame retardants, increase DNA cleavage by TopIIα in vitro and in cultured human cells [85]. Recent CRISPR-Cas9 screening against a large panel of genotoxic agents identified the synthetic small molecule pyridostatin as a Top2 inhibitor. Pyridostatin is a G-quadruplex stabilizer and this stabilization mechanism may lead to Top2 trapping on DNA [38].

4. Flavonols, flavonols, flavones, isoflavones, flavanones, and anthocyanidins act as Top2 poisons and trigger illegitimate DNA repair mechanisms

A catalytic Top2 inhibitor such as dexrazoxane acts to prevent DNA from binding to Top2 thus preventing any part of the catalytic cycle to occur [55–59]. By contrast, some chemicals including bioflavonoids act as Top2 "poisons" (**Figure 5**) [28, 86]. A Top2 poison acts on Top2 after DNA binding and prevents the normal function of Top2 (step 2 of catalytic cycle, see **Figure 2**). Top2 poisons can be further classified as covalent or traditional poisons. The potential as a covalent or traditional poison is dependent on biochemical structure. These groups are not mutually exclusive and individual bioflavonoids can act through one or both mechanisms [29, 86].

4.1 Bioflavonoids as covalent Top2 poisons

Flavanols, flavonols, flavones, flavanones, and anthocyanidins (but not isoflavones) have the potential to act as strong covalent Top2 poisons [86]. A covalent Top2 poison works in a redox-dependent manner, binding to a distal site on the Top2 enzyme and increasing its ability to cause a DSB in step 2 of the catalytic multi-step reaction through conformational changes to the enzyme. The key structural component for a covalent poison is having 3 –OH groups on the B ring of the bioflavonoid structure. However, it is likely bioflavonoids with 2 –OH groups on the B ring act as a weak covalent poison and the ability to act as a covalent poison increases with more –OH groups (**Figure 5**) [49–50]. A 4'-OH group on the B ring is necessary for binding, and 3′ and 5'-OH groups improve covalent binding strength. Thus, a strong covalent poison contains 3 –OH groups on the B ring of the bioflavonoid structure. For example, among the flavonols, structure predicts that myricetin has high activity, quercetin has intermediate activity, and kaempferol has weak activity, if at all, as a covalent Top2 poison (**Figure 4**). Cell free studies support this and show that myricetin as well as epigallocatechin-gallate (ECGC) act as strong coalvent poisons, quercetin acts as a weak traditional poison, but kaempferol does not have this activity [29, 87].
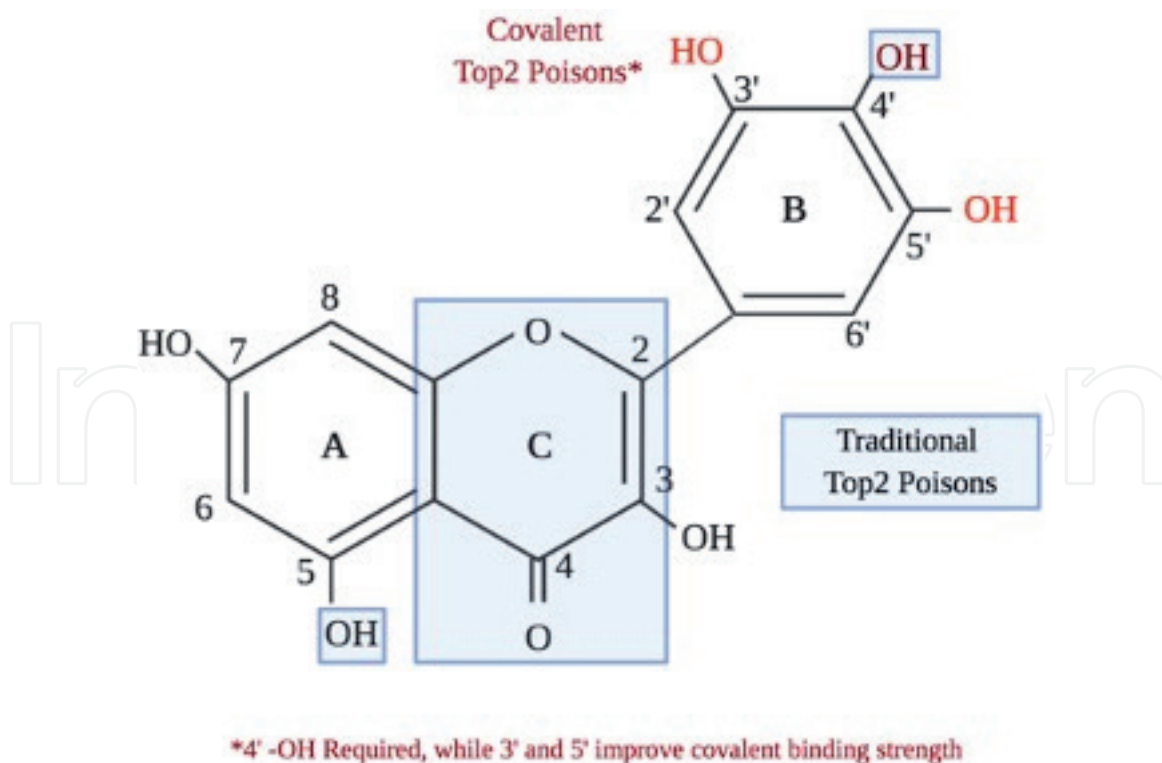
Figure 5.
Bioflavonoid classification as a covalent or traditional topoisomerase II poison. The blue boxed regions indicate required biochemical features for a traditional Top2 poison. The red 3', 4' and 5'-OH groups on the B ring are necessary for covalent Top2 poisons. The 4'-OH group is required for covalent binding, while the 3', 5' increase the binding affinity, therefore a bioflavonoid with all 3-OH groups would be a strong covalent poison.

4.2 Bioflavonoids as traditional Top2 poisons

Flavones, flavonols, isoflavones, and flavanones (but not flavanols or antho-cyanidins) have the all act as traditional (or interfacial) Top2 poisons. The key structural components for a traditional Top2 poison are a 5'-OH group in the A ring, a 4'-OH group in the B ring, and a 4' = O in the C ring (**Figure 5**). A traditional (or interfacial) Top2 poison stalls the enzyme by binding to the active site of the enzyme preventing religation, thereby resulting in the formation of a stabilized cleavage complex (SCC) [88, 89]. Flavonols are strong traditional poisons and both cell free and cell culture systems support this. Similarly, experiments in cell culture systems examining the kinetics of DSB repair following exposure to acute doses of bioflavonoids support the model that flavonols, flavones, and isoflavones including kaempferol, quercetin, myricetin, genistein, and luteolin and each act as a traditional Top2 poison. However, combinatorial activity of genistein, quercetin and luteolin together suggests they may have weak covalent poisoning capabilities when they have to compete for the traditional poisoning binding site [29].

4.3 Bioflavonoids trigger illegitimate DNA repair mechanisms

Bioflavonoids with either covalent and traditional Top2 poisoning activity induce the DSB-mediated DDR as evidenced by induction of γ-H2AX foci, ATM phosphorylation, and p53 signaling [90–92]. However, a more direct role or influence of these compounds on the repair of damage is not as clear [93]. Acute doses induce DNA damage and DDR as detected by γ-H2AX foci and phosphorylation of ATM in stem cells and CD34+ hematopoietic progenitor cells [94, 95].

Genistein and quercetin inhibit Top2 to induce DNA DSBs, and also appear to influence DSB repair pathway choice. Protein level analysis for HR, C-NHEJ, and Alt-EJ specific proteins suggests that genistein and quercetin suppress HR by reducing BRCA2 and Rad51 expression, as well as suppress C-NHEJ by suppressing levels of DNA-PKcs, Ku80, XLF and XRCC4 and trigger Alt-EJ by increasing levels of CtIP and Polymerase θ [96, 97]. DNA reporter assays suggest that quercetin interferes with DNA repair mechanisms such as HR and C-NHEJ by inhibition of PI3K/Akt signaling. In support of these studies, exposure to multiple bioflavonoids promotes the generation of chromosomal translocations in a dose-dependent manner [29, 87].

Bioflavonoids that have traditional Top2 poisoning activity lead to trapped SCCs on the DNA. Removal of SCCs is performed by the small ubiquitin-related modifier ligase ZNF45/tyrosyl-DNA phosphodiesterase 2 (ZATT/TDP2) complex. Removal of the SCC is required for DSB repair by C-NHEJ. If ZATT/TDP2 does not remove the SCC, the MRN complex or CtIP with nuclease activity may resect the DNA ends with the SCC attached to allow for DSB repair by HR or Alt-EJ [55, 94, 98–101]. Inhibition or mutation of multiple DNA repair proteins potentiates cytotoxicity of Top2 inhibitors, and MRE11 plays a direct mechanistic role in removal of Top2-DNA complexes in yeast and mammals [102, 103].

5. Pleiotropic effects of bioflavonoids

Due to their antioxidant capacity, bioflavonoids are included in dietary supplements for their presumed health benefits in protecting against inflammation, cardiovascular diseases, and cancer [87]. These beneficial health properties are due to the number of pleiotropic effects bioflavonoids have on cells by impacting signal transduction pathways, DSB repair and the cellular epigenetic landscape, which can lead to protein level changes, cell cycle stalling, and apoptosis [16, 69].

5.1 Bioflavonoids and signal transduction pathways

Bioflavonoids have antioxidant and anti-inflammatory properties. Their antioxidant properties are due to their ability to reduce reactive oxygen species of the multiple –OH groups in their chemical structure. Their anti-inflammatory properties are due to their interference with signal transduction pathways and down-regulation in the production of pro-inflammatory cytokines. Bioflavonoids decrease inflammation and immune cell recruitment through interference with the ERK/MAP kinase and NF-κB signal transduction pathways which can be beneficial to human health. NF-κB is a transcription factor that upon activation is transported into the nucleus and binds to the promoter region for a number of cytokines and apoptotic genes; therefore reduced pathway activation leads to lower pro-inflammatory cytokine production and increased cell survival [104]. Extracts from the plant *Ginkgo biloba*, rich in bioflavonoids, act as an herbal antioxidant, augment the transcription of TNF-α causing reduced activation of the NF-κB pathway. Apigenin has shown similar down regulatory effects on cytokine production likely through the modulation of NF-κB activation [105]. Quercetin and fisetin inhibit pro-inflammatory cytokine production through the suppression of NF-κB activation by decreased phosphorylation of extracellular signal-regulated (ERK) kinase and p38 mitogen-activated protein (MAP) kinase that are activators of NF-κB [106–108]. Myricetin has been shown to affect the phosphatidylinositol 3-kinase (PI3-K) pathway inducing apoptosis in pancreatic cells [109].

5.2 Bioflavonoids and epigenetic modifications

Studies in cancer cell lines demonstrate epigenetic modifications caused by bioflavonoids. Genistein, quercetin, curcumin, EGCG, hesperidin, and naringin are inhibitors of DNA methyltransferases leading to hypomethylation of DNA. In addition, many of these bioflavonoids have also been shown to act on histone acetyltransferases and histone deacetyltransferases causing cell wide alterations in histone epigenetic modification patterns [109].

Long-term epigenetic effects of bioflavonoids compounds were addressed in several mouse model studies. Exposure to genistein through maternal diet during pregnancy can have long-lasting effects on the progeny. In agouti mouse pups exposed to genistein from conception until birth, epigenetic changes were observed as altered coat color, as well as significant downregulation of genes involved in hematopoiesis of bone marrow cells, increased erythropoiesis, and a permanent signature hypermethylation of repetitive elements in hematopoietic lineages [110]. Likewise, in mice exposed to quercetin from conception until birth resulted in upregulated iron-associated cytokine expression, significantly increased iron storage in the liver, and hypermethylation of repetitive elements. Epigenetic modifications lead to long term gene expression changes of cytokines associated with inflammation in the liver of the mice in adulthood [111, 112].

6. Implications for human health

6.1 Potential anti-cancer applications

While bioflavonoids can be beneficial through intake at low or moderate doses, high doses and acute exposure of bioflavonoids may more drastically inhibit Top2 and impact genome integrity and cell survival, thus changing their overall impact on cells and human health. *In vitro* studies support the idea that bioflavonoids genistein and quercetin may act as chemo-preventive or anti-cancer agents by altering major processes within cancer cells such as apoptosis, cell cycle, angiogenesis and metastasis [113, 114]. Genistein has synergistic behavior with well-known anticancer drugs adriamycin, docetaxel, and tamoxifen, suggesting a potential role in combination cancer therapy [78]. Quercetin in combination with doxorubicin was found to be more effective in inducing apoptosis within the SKOV-3 cells [114]. A combinatory treatment with quercetin and curcumin synergistically induce anti-cancer activity in triple-negative breast cancer cells by modulating tumor suppressor genes in particular enhancing BRCA1 expression [115].

Several bioflavonoids have been investigated as alternate cancer therapeutics that are less genotoxic than traditional chemotherapeutics but equally effective. High concentrations of myricetin causes Top2-mediated DNA damage and apoptosis in K652 cells [116]. Fisetin interrupts the MAPK-dependent NF-κB signaling pathway in cervical cancer cells, inhibiting migration and invasion [114]. Several *in vitro* and *in vivo* studies indicate that luteolin can suppress metastasis of breast cancer by reversing epithelial-mesenchymal transition, or by acting as an antiangiogenic therapeutic inhibiting VEGF production and suppressing invasion [117, 118].

While these observations strengthen the notion that flavonoids could be useful anti-cancer agents, to date minimal clinical studies have demonstrated that these bioflavonoids retain anti-cancer properties in humans *in vivo*. A Phase I study/ pharmacokinetic trial of quercetin in cancer patients intravenously injected quercetin in 11 patients with cancer at varying doses of 60–2000 mg/m^2 and identified 945 mg/m^2 as a safe and effective dose [119].

6.2 Potential inducers of infant leukemia

Aberrant repair of DNA DSBs caused by either endogenous or exogenous agents has the potential to result in DNA sequence mutations or genome rearrangements such as chromosomal translocations which can lead to disease. Negative consequences of high bioflavonoid intake can be observed most prominently in pregnant women. Epidemiological data from countries whose citizens have higher bioflavonoid intake (particularly soy products) had a 2–3 times higher incidence of infant leukemia, characterized by chromosomal translocation, suggesting maternal intake of high amounts of bioflavonoids could lead to this particular genome rearrangement and infant leukemia [120].

Infant leukemia typically occurs due to translocation events involving the mixed lineage leukemia (*MLL*) gene. Most of the *MLL* rearrangements observed in patients with infant leukemia and therapy-related leukemia (tAML) cluster together in a well-defined region of the *MLL* locus [121]. tAML is associated with treatment with Top2 poisons etoposide or doxorubicin [86, 88, 121] which has led to the hypothesis and working model that ingestion of natural Top2 poisons including bioflavonoids can lead to these translocation events and tumorigenesis [121, 122]. In support of this, boflavonoids have been shown to inhibit Top2 and induce *MLL* cleavage and translocations in hematopoietic stem cell-enriched populations [87, 121].

Foods contain multiple different bioflavonoids, and bioflavonoids are bio-accumulative which likely increases plasma concentrations [123]. Study of the potential for environmental or dietary compounds to induce infant leukemias is more relevant since they cross the placental barrier as shown with the synthetic bioflavonoid EMD-49209 [124], genistein [111, 125], quercetin [111], herbal medicines, dipyrone, and pesticides including the mosquitocidal Baygon [126, 127]. Genotoxic effects of quercetin on the human hemopoietic stem and progenitor cells (HSPCs) were shown using a genetically engineered placental barrier model from a specialized human cell line. This study showed that approximately 10% of quercetin from the maternal side is capable of crossing the placental barrier and accumulating in the fetus. Exposure *in utero* is likely more damaging due to differences in metabolic and excretion rates of mother and fetus [128] as well as rapidly developing and proliferating fetal cells that are more sensitive to Top2 inhibiting agents [129].

7. Conclusion

Bioflavonoids are prevalent in the human diet from natural sources such as fruits and vegetables, but are also found at supranatural concentrations in dietary supplements and energy drinks. These chemical compounds have numerous cellular effects including interfering with signal transduction pathways, modifying the DNA damage response and epigenetic markers, and poisoning of Top2 causing DNA DSBs and leading to aberrant repair. Given the number of cellular pathways bioflavonoids affect, and the DNA damage caused by bioflavonoid exposure, it is possible that bioflavonoids could be used as natural analogs of traditional chemotherapeutic agents. However, more research is needed to understand how these bioflavonoids cause DNA damage through Top2-dependent or -independent pathways to understand potential off-target negative effects. In addition, further research will be needed to understand the dose-dependent activities of bioflavonoids and at what doses they may be chemo-protective versus what threshold doses they may induce DNA damage that is mutagenic, and finally at what high acute doses they may induce DNA damage and apoptosis to act as effective alternative to traditional chemotherapeutic agents.

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Conflict of interest

The authors indicate no conflict of interest.

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