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Flavonoids and genomic instability induced by ionizing radiation

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DNA is the cellular target that has the most damage induced by ionizing radiation (IR). If genomic instability resulting from this DNA damage is not correctly repaired, it leads to mutation, cancer and cell death. Flavonoids are a family of natural products that affect oxidative stress and enhance genomic stability through DNA interaction. Although flavonoids exert protective effects against IR in normal cells, they enhance genotoxicity effects of this radiation in cancer cells, a beneficial effect that is of interest in the design of new anticancer pharmaceuticals. This review describes the molecular effects of IR on DNA structure and mechanisms by which flavonoids exert their effect on ionizing-radiation-induced genomic instability.

Introduction

In normal cellular metabolism, reactive oxygen species (ROS) are produced, but their effects are balanced by the endogenous repair system. ROS levels can be elevated by exposure to oxidative stress such as ionizing radiation (IR; e.g. X-ray, gamma ray, β^-, α or proton) or by deficiencies in the cellular repair process. Exposure to ionizing irradiation results in the production of free radicals and toxic substances that can damage crucial macromolecules – including DNA, cell membranes and enzymes – and can cause cell death. DNA damage includes genotoxicity, chromosomal abnormalities, gene mutations and cell death [1]. Double-strand breaks (DSBs) induced by IR are considered the most dangerous lesion for cell survival and induction of genomic instability. Single-strand breaks (SSBs), which appear not only as a direct result of radiation but also during nucleotide excision repair of DNA or during normal replication, can be converted into DSBs if they are not rejoined. Flavonoids, a family of natural products present in many types of fruits and herbs, have many pharmacological properties including anti-inflammatory, hepatoprotective and antioxidant activities [2] and are able to interact with free radicals and substances produced by oxidative stress. These interactions are facilitated by their structure of polyphenol rings with hydroxyl and/or methoxyl groups, which can specifically bind with a base or other groups in the DNA backbone, as well as trap ROS. These polyphenolic compounds considerably mitigate the effects of ionizing irradiation at the molecular, cellular and/or tissue level [3–5].

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Throughout life, humans are exposed to IR from numerous sources including natural background sources, diagnostic procedures and the treatment of cancer by radiotherapy. Humans also consume daily high amounts of flavonoids through fruit and vegetable intake. This review focuses on how the chemical structure of flavonoids interacts with free radicals and stabilizes DNA structure.

Reactive substances produced by IR

IR can remove electrons from atoms to form ions. The extent of these ionizations is dependent on the type of radiation and its energy. IR affects cellular crucial macromolecules directly and indirectly. Although the direct effect observed during exposure to IR is very small, the effect is more likely to happen with high-energy radiation (mostly particles) than photon. Direct radiation, rarely from IR but more commonly from high-energy particle radiation, can interact directly with the atoms and break bonds in a molecule such as DNA. Because the body and its cells are composed primarily of water, there is a much higher probability of radiation interacting with this molecule. When this occurs, it can break the intramolecular bonds to form ionized water and produce highly reactive free radicals such as hydrogen ($H^{\bullet}$) and hydroxyl ($HO^{\bullet}$). The combination of these free radicals can form toxic substances such as hydrogen peroxide (H_2O_2), which can contribute to the destruction of the cell. Whereas $H^{\bullet}$ and $HO^{\bullet}$ have short life spans, the longer life span of H_2O_2 enables it to migrate to sites distant from the point of IR exposure [6,7]. IR can also generate reactive nitrogen oxide species as toxic products, which are involved in radiation-induced signalling mechanisms [7]. These toxic substances produced by indirect effects are the main reason for the side-effects of IR. Interactions of free radicals with organic molecules can produce free radical residues that subsequently transfer damage to macromolecules and thus contribute to cumulative damage. The phenyl ring of tyrosine is a common target for free radical and oxidative substance attack, and this results in a long-living tyrosine phenoxyl [8]. Radiation-stimulated ROS and reactive nitrogen oxide species have been shown to activate signal transduction cascades such as mitogen-activated protein kinase to initiate through events in the plasma membrane, the cytoplasm and the nucleus, resulting in toxic and protective responses in cells [1,6].

Effect of ionizing irradiation on macromolecules and DNA

DNA, RNA, enzymes, membrane and cellular proteins can suffer considerable damage from IR. Hydroxyl radicals produced by IR [9] oxidize amino acids, resulting in the hydroxylation of aromatic and aliphatic side chains and the addition of other groups on aromatic rings. This can induce progressive alterations in the structure of peptides, including cross-linkage and fragmentation or even alteration to individual amino acids, which change protein functions such as enzymatic activity. Oxidative reactive substances can produce reactive moieties in the protein structure that can interact with other peptides or molecules such as fatty acids and carbohydrate derivatives and lead to cross-linking of the protein with other macromolecules. These protein–macromolecule links are inactive biologically [10,11]. Hydroxyl radicals seem to play an important part in the formation of DNA–protein cross-links in chromatin [12]. Moreover, reactive moieties of proteins can be coupled to the damage of other biomolecules, such as lipids, leading to effects on physiological cell activities [13,14].

There is considerable evidence – primarily from the lethal effects of radioactive compounds that accumulate in the nucleus rather than cytoplasm or plasma membrane – suggesting that DNA is the primary target for cell damage from IR [15,16]. IR and ROS interactions can result in changes in the deoxyribose ring and base structures [17], intra- and interstrand DNA–DNA cross-links, DNA SSBs and DSBs, and DNA–protein cross-links [15]. Each of these changes is discussed in more detail below.

Changes to DNA bases and sugars. Hydroxyl radicals react with nucleotide bases such as thymine and cytosine by either adding a double bond or abstracting a hydrogen atom. Both reactions lead to the production of reactive intermediates (Fig. 1). Damage to DNA bases can destabilize the bridge between the base and sugar, with loss of the base moiety and the formation of basic deoxyribose residues. Hydroxyl radicals can abstract hydrogen atoms from the sugar–phosphate backbone of DNA, which generates 2-deoxyribose radicals that attack molecular oxygen or thiols, leading to strand damage. Sugar radicals (Fig. 1) might result in the release of an unchanged base from DNA [18] and can also react with purine or pyrimidine residues on the same nucleotide to yield an interstrand nucleotide cross-link (Fig. 1) [19]. Free radicals can also attack the guanine base to produce a purine ring radical (Fig. 1). This process can lead to an open ring pyrimidine, resulting in the destruction of the guanine base [19,20].

Intra- and interstrand DNA–DNA cross-links. The coupling of methylcytosine or thymine radicals to a neighbouring purine base produces an intrastrand cross-link lesion [21]. Pyrimidine-derived radicals have a more important role than other secondary radicals in inducing intrastrand cross-links [20,22]. Furthermore, there is evidence that the repair of interstrand cross-links involves the generation of a DSB during the unhooking process. These lesions, therefore, are extremely cytotoxic and mutagenic. Finally, a nucleotide radical produced by IR can be covalently bound to the deoxyribose of the same nucleotide or to its neighbouring base to yield cyclonucleosides and nucleobase–nucleobase inter- or intrastrand cross-links, respectively. These alkyl radicals react with other bases, resulting in interstrand cross-linked DNA [23]. Biochemical studies have demonstrated that these lesions markedly block DNA replication and transcription if not repaired.

The most cytotoxic DNA lesion is a DSB, and more specifically, a single unrepaired DSB. The religation of a DSB is crucial for cell survival, and SSBs and DSBs are mostly repaired by cellular repair machinery. If repaired improperly, a DSB can potentially result in chromosome aberration and might lead to genetic instability, mutation and chromosome rearrangements [24,25]. If DNA damage is effectively repaired and the cell is capable of proliferation, then the progeny of such an irradiated cell is normal. If the damage is not lethal and is misrepaired or unrepaired, however, then the progeny of a single irradiated cell would be expected to show radiation-induced genetic changes in all descendant cells. This can result in mutation and cell death. These lethal, mutagenic or clastogenic effects are outcomes of radiation-induced genetic instability (Fig. 2).

DNA–protein cross-links. These are harmful to cells because protein immobilized on DNA strands can block normal DNA transcription and replication in cells.

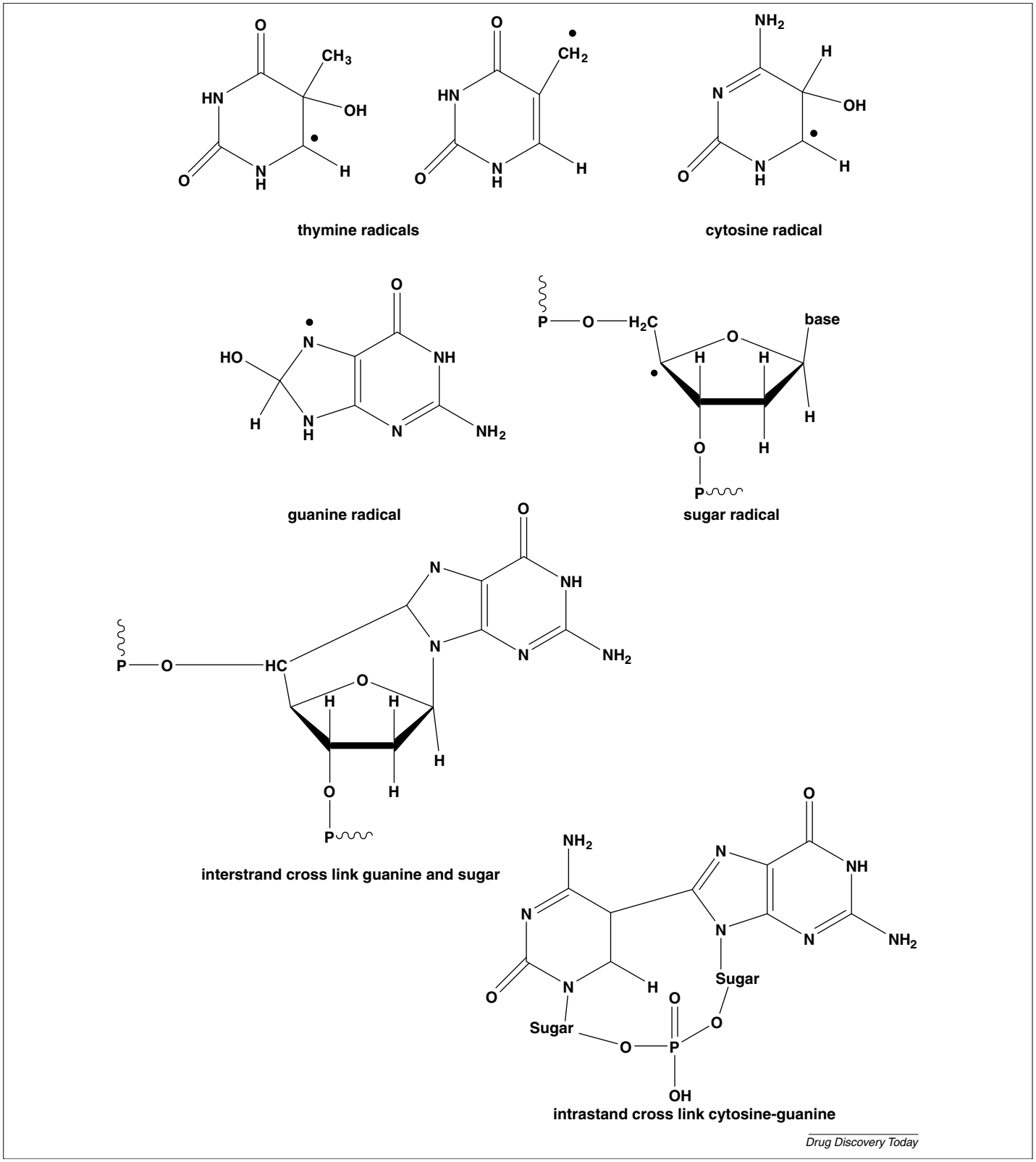

FIGURE 1

Base and sugar radicals produced by IR.

Cellular defence system

DNA damage occurs naturally by several means. Normal cellular metabolic processes generate ROS that can attack DNA to produce a variety of potential DNA lesions. The body is equipped with several means of mitigating and removing the effects of free radicals on crucial molecules. These mechanisms constitute the cellular defence system, which acts before free radicals can attack molecules and which is involved in the repair of SSB and DSBs

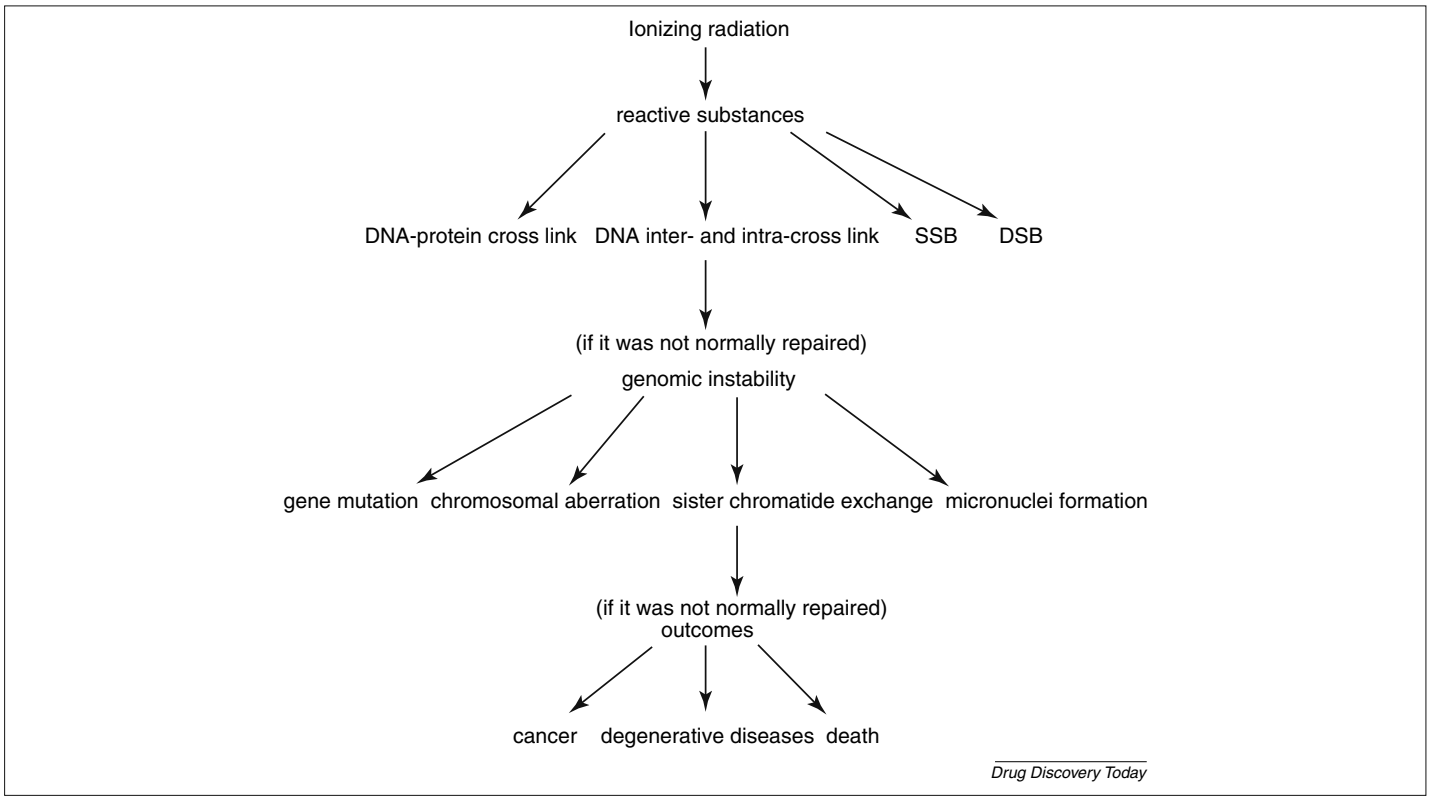

FIGURE 2

Different types of genomic instability induced by ionizing radiation and their outcomes.

through enzymatic pathways. The antioxidant and antioxidant-related enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase comprise the cellular defence mechanism responsible for the inhibition and mitigation of ROS attacks on biomolecules. SOD is the most effective of these enzymes in protecting cells against IR-induced damage [26]. Animal treatment with SOD [27], overexpression of SOD [28] or use of compounds that mimic SOD (e.g. Mn-porphyrin) [29] resulted in considerably radioprotective effects and reduced radiation-induced DNA damage. The most abundant cellular antioxidant is the tripeptide L-γ-glutamyl-L-cysteinylglycine (GSH). GSH prevents oxidation either directly, by scavenging reactive oxygen radicals through its thiol group, or indirectly, by utilizing γ-glutamyl transferase enzymes. The cellular GSH pool helps maintain the redox balance to protect against oxidative stress [30]. This plays a crucial part in the inhibition and recovery of cross-linked proteins and other macromolecules under oxidative stress. The radioprotective effects of exogenous antioxidants, such as melatonin and lycopene, have been attributed to increased GSH levels and increased γ-glutamyl transferase activity within cells [31,32].

Chromatin DNA is tightly packaged in a complex with histone and non-histone proteins. This DNA–protein complex protects DNA from free radical attack [18,33]. The removal of DNA-bound protein from chromatin results in an increase of radiation-induced DSBs [34]. Histone lysine methylation has been linked to the activation of a series of DNA repair enzymes [33].

Endogenous repair systems are involved in the chromatin reorganization and repair of damaged bases and strand breaks. Damaged bases are excised by a combination of DNA glycosylases, which catalyse the hydrolysis of *N*-glycosylic bonds linked to chemically altered bases, and endonucleases, deoxyribosephosphodiesterases and exonucleases. The gap is finally filled by DNA polymerase and the strand is sealed by DNA ligase [35]. Major DNA repair pathways include the DNA non-homologous end-joining pathway, the homologous recombination pathway and response pathways such as the ataxia telangiectasia and Rad3-related pathway. IR induces response pathways in which enzymes such as Ku, Xrcc4 and Rad51 are involved in DSB repair [36,37].

Exogenous radiation protective agents

Several different mechanisms have been proposed for radioprotection through exogenous compounds, including direct scavenging of ROS, hydrogen donation to reactive free radicals, inducing and/or altering the levels of endogenous enzymes for detoxifying ROS, increasing DNA stability, lowering the production of ROS by the induction of hypoxia with consumption of local oxygen, and enhancing the DNA damage repair pathway.

The primary mechanism of action of radioprotective agents is ROS scavenging. Because genomic instability is mainly induced by ROS produced by IR, a compound with sufficient reactivity toward a ROS can intercept the ROS free radical before it has an opportunity to attack crucial molecules such as DNA. Thiol-containing compounds, in particular, have an excellent radioprotective effect. Cysteamine and dimethyl sulfoxide (DMSO) both reduce genomic instability, an effect mainly related to the inhibition of ROS. Cysteamine has a greater protective effect than DMSO because of its positive charge [38], which enables it to interact electrostatically with the negatively charged sugar–phosphate backbone

of the DNA. This interaction acts to position the thiol in a location where it can react with ROS before they can attack the DNA [39]. Other radioprotective thiol-containing synthetic compounds include Amifostine, an FDA-approved drug for the protection of patients against radiation-induced xerostomia, and WR1065, an active metabolite that protected cells from chromosomal damage and cell death [40]. Like the synthetic antioxidants and radioprotectors (e.g. amifostine and cysteamine), antioxidants derived from natural sources also protect against DNA damage and cell death induced by IR. Natural compounds such as vitamin E [41], melatonin [42] and herbal medicine (e.g. citrus and hawthorn extracts) [43,44] have also been shown to exert radioprotective effects by free radical scavenging. Radioprotective agents are categorized based on their mechanism of action (i.e. antioxidant and immunostimulator), origin (i.e. synthetic and natural, compounds) and/or chemical structure (i.e. thiol and bisbenzimidazole ring-containing compounds). This categorization is not exact, and radioprotectors can have overlapping mechanisms and structures. Because the use of radioprotective agents can be crucial in reducing side-effects induced by IR in patients undergoing radiotherapy or in personnel in radiological workplaces, many compounds have been evaluated as radioprotective agents in cell culture, animal and human experiments over the past 60 years. There are several excellent reviews to describe the history, mechanisms and types of these compounds with potential radioprotective properties [3–5,45,46].

Flavonoids

Flavonoids are a family of natural products with a polyphenolic structure that are found in plants. More than 4000 flavonoids have been identified, many from fruits, vegetables and beverages (tea, coffee and fruit drinks). For example, apple contains the flavanols quercetin, rutin, epicatechin and catechin [47]. The daily intake of flavonoids is different in each country, reflecting differences in diet, especially the consumption of tea. High consumption of tea might be the most influential factor in total flavonoid intake in certain groups of people. This intake is high compared to the average daily intake of other dietary antioxidants such as vitamin C, vitamin E or carotenoids.

Flavonoids have a unique structure based on three phenyl rings, A, B and C (Fig. 3). Ring B can bind to position 3 at fused ring C to form isoflavone. The preferred glycosylation site on flavonoids is the 3 position, and less frequently the 7 position, with glucose the most common sugar residue. Flavonoids are categorized into the following groups based on their chemical structure: flavonols (e.g. quercetin), flavones (e.g. apigenin), flavanones (e.g. hesperetin), isoflavones (e.g. genestein) and flavanols (e.g. catechin). The chemical structure of flavonoids are dependent on hydroxy group at position C2, C2═C3 double bond, 4-carbonyl group and steric conformation of B ring (Fig. 3). The biological effects of flavonoids depend upon their chemical structure. The position of hydroxyl groups and other features are important for their antioxidant and free-radical-scavenging effects. Because of their considerable health-related effects and their unique chemical structure, these bioactive compounds are the subjects of much medical and biological research as investigators try to find more biological properties and mechanisms. Extensive studies of flavonoids have shown that these compounds not only have antioxidant [48] and radioprotective properties but also have antibacterial [49], inflammatory [50], antioxidant [48], anticancer [51] and antigenotoxic [52] activities. These biological effects of flavonoids are attributed mainly to their antioxidant effects. Whereas several human studies have shown ingestion of flavonoids to have health benefits in preventing cancer [53,54], other studies have not found this to be the case [55,56].

In contrast to their beneficial effects, some flavonoids have also been found to be mutagenic and genotoxic *in vitro*. Several studies reported that quercetin has genotoxic effects in cell culture and microbial assays [57–59]. By contrast, *in vitro* studies using Salmonella as a microbial assay did not indicate that quercetin had any mutagenic effects [60]. In addition, quercetin did not show any genotoxicity in an *in vivo* model [59]. Short- and long-term administration of a quercetin-supplemented diet in animals did not result in any carcinogenicity and genotoxicity [59,61]. Harwood *et al.* [62] recently reviewed *in vitro* and *in vivo* studies related to probable genotoxicity induced by quercetin and concluded that no toxicologically and mutagenically considerable adverse effects were reported when food-grade quercetin was added to foods at levels approximating intakes of naturally occurring quercetin in the diet of consumers with a high fruit and vegetable intake (i.e. 200–500 mg/day). Quercetin, therefore, has been classified as non-carcinogenic to humans and is safe for human usage, with quercetin supplements commercially available [63]. Genistein, a phyto-oestrogen present in high levels in soybean, is a potent inhibitor of type II topoisomerases [64]. Type II topoisomerases are enzymes that unwind DNA and generate DNA strand breaks that have the potential to fragment the genome at every cycle of their action. Although topoisomerases are essential for the survival and proliferation of cells, they also have considerable genotoxic effects. Genistein binds to and stabilizes the topoisomerase–DNA complex, inhibiting religation and resulting in DNA strand breaks [65]. The potentially DNA-damaging or mutagenic effects of genistein that have been suggested in some studies *in vitro* [64,66] are not likely to occur *in vivo*, even at high dietary levels of genistein [67]. This paradoxical effect of genistein is partly related to its dosage level. In 1999, the US FDA recommended the daily ingestion of 25 g of total soy protein [67].

Whereas flavonoids generally demonstrate antioxidant activity, they can shift to prooxidant activity in the presence of transition metals. In addition, although flavonoids directly react with free radicals as antioxidants, chelation of metal ions might involve the production of ROS. Flavonoids can bind to transitional metals ions such as Cu(II) and Fe(III), which are the most redox-active metals in living cells. All types of flavonoids possess three domains able to react with metal ions: the 3,4-dihydroxy system located on ring B, the 3-hydroxy or 5-hydroxy groups of ring C and the 4-carbonyl group in ring C [68] (Fig. 3). The reduction of Cu^{2+} and Fe^{3+} to Cu^{+} and Fe^{2+}, respectively, by phenolic compounds can form superoxide radical anions by a single electron reduction of the oxygen molecule. The superoxide radical is, in turn, converted to hydrogen peroxide and a hydroxyl radical, causing the formation of a DNA base adduct [69]. This prooxidation activity is responsible for the ability of flavonoids to promote cellular toxicity mainly through DNA damage [70]. Copper and zinc are the major metals naturally associated closely with chromosomes, so the presence of Cu^{2+} rather than that of Fe^{3+} is more considerable for DNA damage produced by the prooxidation of flavonoids [71]. The location of

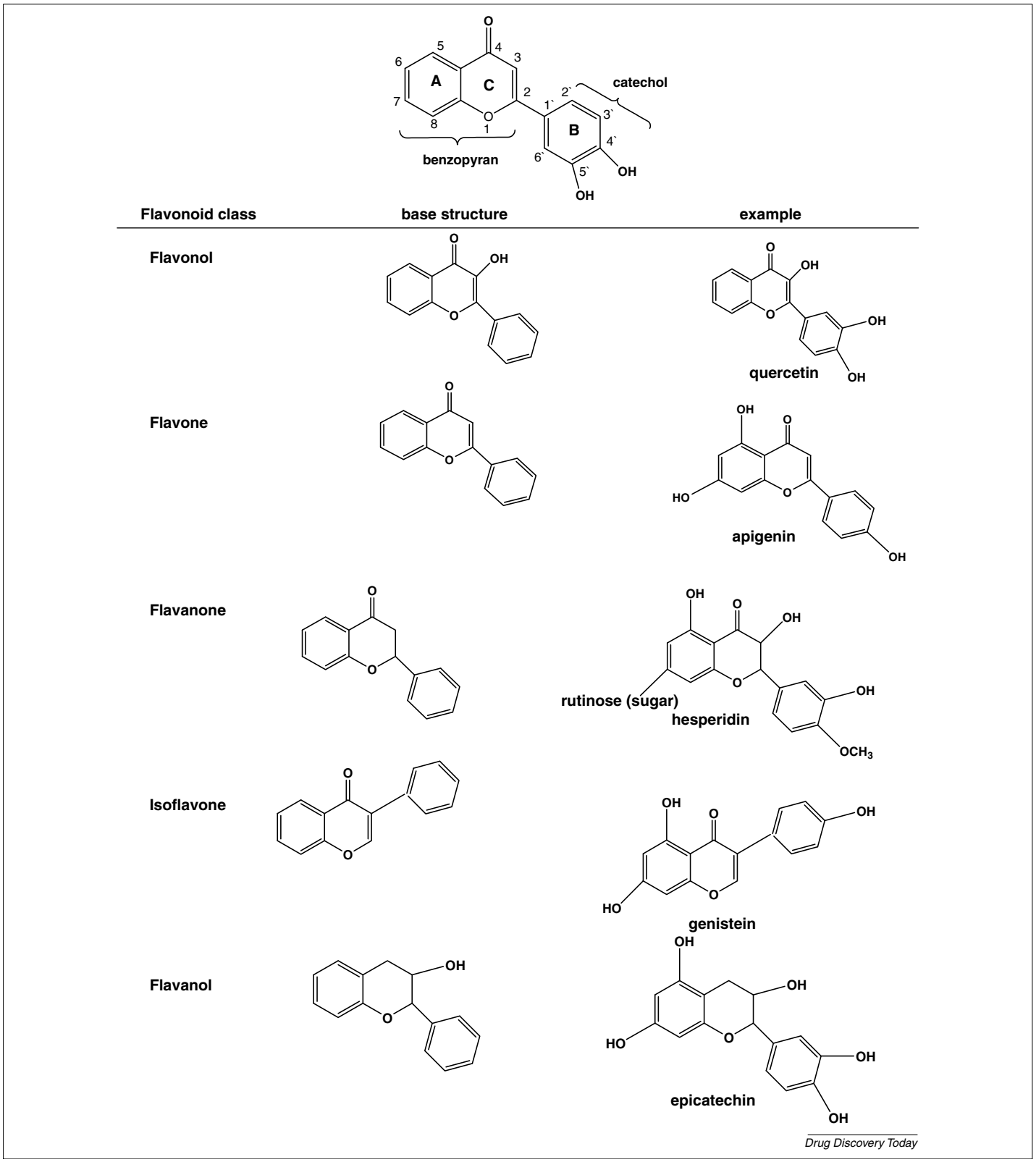

FIGURE 3

Classification, structure and example of the main classes of flavonoids.

redox-active metals is important because they generate hydroxyl radicals and other ROS with very short life spans and penetration through the medium. To have a DNA-damaging effect, these reactive substances must be produced in close proximity to DNA. Both the antioxidant and the copper-mediated prooxidant activities of polyphenolic compounds depend on the number and position of hydroxyl groups [70]. Quercetin undergoes more autoxidation and causes more DNA damage because of the reduction of

Cu(II) to Cu(I) compared to kaempferol and morin. Quercetin binds to Cu(II) in close proximity to DNA to form a DNA–Cu(II)–quercetin complex. Cu(I) and superoxide anions are generated through the autoxidation of the quercetin–Cu(II) complex, resulting in the formation of hydroxyl radicals and hydrogen peroxide that cause oxidation and DNA damage. In the presence of quercetin or Cu(II) alone, no DNA cleavage was observed [72]. The flavonoid–Cu(II)–DNA complex is proposed to be responsible for the carcinogenic and mutagenic effects of flavonoids owing to its ability to generate oxidative DNA damage. These experiments were mainly conducted using isolated cellular DNA, however, and care should be taken in the extrapolation of these results to humans.

Because cancer cells have a higher intracellular copper level, it has been suggested that the flavonoid–Cu(II) complex has more cytotoxic effects in cancer cells than in normal cells [71]. Genistein–metal (Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+}) complexes also had anti-proliferative effects against cancer cells. These complexes inhibited cell growth more than free isoflavone and corresponding metal ions and exhibited a statistically G_2/M-phase arrest for human cancer cell lines [73]. The paradox of the opposing antioxidant and DNA-damaging activities of these flavonoid–metal complexes is further discussed later in this review.

Structure–activity relationships of flavonoids and antioxidant activity

Flavonoids have low oxidation potential (+0.125 V and +0.235 V for quercetin and morin, respectively), which means they are easily reduced by ROS and so have greater free-radical-scavenging abilities than high oxidation potential value [74]. The antioxidant activities of flavonoids vary considerably depending on different backbone structures and functional groups and, likewise, the difference in their ROS scavenging can be accounted for by the variation in the number and type of functional groups attached to the main nucleus. There are several functional groups that contribute to increased ROS scavenging. These include the C2═C3 double bond, the adjacent conjugate with the 4-carbonyl group in ring C and the catechol moiety formed by the 4′,5′-dihydroxy structure of ring B (Fig. 3). The additional presence of a 3- and/or 5-hydroxyl group on rings C and A also increases the efficacy of ROS scavenging [75]. The position and number of hydroxyl groups have an important role in antioxidant activity. It is generally accepted that an increase in the number of hydroxyl groups increases the antioxidant activity of flavonoids; for some compounds, however, the presence of a methoxyl group contributes to negative antioxidant activity and is more important for reducing this effect than increasing the number of single hydroxyl groups on ring B [76]. The ROS-scavenging activities might be closely related to the position rather than the numbers of phenolic hydroxyl groups because the activity of an *ortho*-dihydroxyl group is much higher than that of a *meta*-dihydroxyl group [73].

The antioxidant activity of flavonoids depends, in part, on their ability to delocalize electron distribution, resulting in a more stable phenoxyl radical. The stability of this radical is related to the structural planarity of flavonoids between ring B and the benzopyrane nucleolus, which enables resonance effects [75]. When flavonoids react with free radicals, the phenoxyl radicals produced are stabilized by resonance effects of aromatic nucleus [75]. The presence of a hydroxyl group at the 3 position on ring C increases the planarity, whereas the presence of a methoxyl group at the 3 position inhibits this structural planarity owing to steric hindrance, resulting in lower antioxidant activity [76]. The unsaturation of ring C and subsequent conjugation with the 4-OXO group enables electron delocalization across the molecule that stabilizes the aryloxyl radical [75]. Retention of the catechol type structure in ring B and removal of the C2═C3 double bond in ring C eliminates delocalization of electrons from the aryloxyl radical on ring B to ring A. The antioxidant activity of flavonoids is also affected by glycosylation, which reduces their activity. For example, rutin, a glycone quercetin, has lower antioxidant activity than unglycosylated quercetin [77]. Qurecetin contains almost every functional group required for antioxidant activity and so is more potent than other flavonoids such as catechin and hesperidin, which lack some of these functional groups in their structures [75].

In vitro *and* in vivo *experiments on the effects of flavonoids on radiation-induced genetic damage*

Two different aspects of the biological effects of flavonoids have been assessed: increased tumour cell death and preventive effects in normal cells during co-administration of flavonoids and IR.

Several studies have shown different effects of flavonoids on normal and cancer cells during radiation exposure. Genistein and quercetin are known to have multiple cellular effects such as protection of normal cells from DNA damage, arrest of the cell cycle at the G_2/M phase in human cancer cell lines, induced apoptosis and cell death [78,79]. Quercetin-induced apoptosis in tumour cells is associated with caspase activation and suppression of survivin and Bcl-2 [80,81]. In animal models, genistein has immunomodulating and radioprotective properties. Administration of genistein considerably increases the survival rate in gamma-irradiated normal mice by accelerating neutrophil and platelet recovery and hematopoietic progenitor cell reconstitution [82,83]. In different cancer cells, low concentrations of genistein-induced expression of the major cell cycle inhibitory proteins (p53, p21, Bcl-2 and survivin) lead to an increase in radiation-induced genotoxicity. Genistein also stimulates irradiation-induced intracellular ROS production in cancer cells and induced additional apoptosis when cells were subjected to IR [84–86]. This radiosensitivity of genistein is beneficial for the protection of normal cells yet detrimental to cancer cells in patients undergoing radiotherapy. Epigallocatechin-3-gallate (EGCG), a major antioxidant polyphenol found in green tea, has anticarcinogenic and antigenotoxic properties. This polyphenol sensitized the glioblastoma cancer cell response to IR. EGCG antagonized the IR-induced expression of survivin, a cell survival protein, resulting in the arrest of the cell cycle at the G_2/M phase and inhibition of cell growth [87]. The concentration of EGCG is probably a key factor in determining whether EGCG serves to protect or to damage tumour cells during cancer therapies because it induces apoptosis at lower concentrations, whereas it does not accelerate ROS formation. EGCG is able to protect salivary gland cells from damage caused by radiation therapy; however, EGCG might also protect tumour cells during radiotherapy when EGCG concentrations are at physiological levels [88]. In summary, flavonoids can induce an increase in tumour cell toxicity during exposure to IR. This effect is not related to ROS scavenging but is associated with the sup-

pression of cellular proteins such as survivin and with the promotion of cell cycle arrest at the G_2/M phase. These effects cumulatively enhance cell apoptosis induced by IR.

Flavonoids similar to nutritional antioxidants such as vitamin E have beneficial effects on radiation-induced cell damage [89]. Flavonoids have been widely shown to have a protective effect on genomic stability. Several chromosomal and genomic methods have been used to assess these protective effects and are discussed below. Evidence for the formation of IR-induced strand breaks in plasmid DNA comes from evaluation of the degree of DNA supercoiling assessed by gel electrophoresis. Pretreatment of plasmid DNA with quercetin [90] or epicatechin [91] decreases the toxic effects of gamma irradiation by inhibiting DNA strand breaks. The comet assay, or single-cell gel electrophoresis technique, is a sensitive method that measures SSBs of DNA for the evaluation of genotoxicity induced by IR or toxic substances. Treatment of irradiated mice with flavonoids considerably decreases the level of primary DNA damage (SSBs). This protective effect is observed for naringin, chrysin [92], epicatechin [91] and troxerutin [93]. Micronuclei (Mn) formation, which assesses the degree of DSBs in DNA, is one of the best assays for the evaluation of genome instability induced by IR. This method is performed *in vitro* (e.g. in human cultured lymphocytes) and *in vivo* (e.g. in mouse bone marrow cells and peripheral blood leucocytes). Higher genome instability results in a higher frequency of Mn. Pretreatment of human culture lymphocytes with flavonoids statistically significant reduced the Mn frequencies that were increased by IR. Quercetin [90], hesperidin [94,95], swertisin [96] and troxerutin [93] statistically significant decrease the frequency of Mn induced by IR in human binucleated lymphocytes, an effect potentially leading to reduced genomic damage. These flavonoids did not increase the frequency of Mn in non-irradiated human lymphocyte cultures treated at IR protective doses. Flavonoids including troxerutin [93], naringin [97] and hesperidin [98] have protective effects against genotoxicity induced by IR in mice, as assessed by Mn assay.

It is clear that flavonoids reduce cell death owing to their antioxidant effects and protective effects on biomolecules such as DNA. In animal studies, administration of several flavonoids such as morin [99] and genistein [100] statistically significant reduced mortality rates induced by IR. These effects are thought to be related to the protective effects of flavonoids and maintenance or recovery of levels of crucial cell populations such as platelets, white blood cells and hematopoietic progenitor cells, which are reduced by IR [5].

Mechanisms related to flavonoids and genomic stability

Free radical scavenging

Because approximately 65% of DNA damage is caused by the indirect effect of free radicals such as $HO^{\bullet}$ that are produced from the radiolysis of surrounding water molecules, a major role of flavonoids is scavenging these free radicals. Their protective effect in cells is attributed to the inhibition of ROS before they are able to attack DNA and other macromolecules. Because ROS are mostly short-lived, they have to react with DNA immediately after their production by IR [101]. Flavonoids can donate a proton from their hydroxyl groups to the free radical, resulting in free radical repair and an inert molecule. The phenoxy radicals produced are stabilized by delocalization of the unpaired electron within the aromatic structure. The *o*-dihydroxy group in ring B and 3-hydroxy along with 4-OXO and C2═C3 double bond on benzopyrane ring attribute particularly for stabilization of phenoxy radical induced by ROS. Therefore, these polyphenols are excellent scavengers of free radicals because of the high reactivity of their hydroxyl substituents and the resonance effects of their unique polyphenyl [102]. It is estimated that high *in vitro* antioxidant activity is also related to high radioprotective effects for polyphenols. Grape seed extract, which contains high levels of poyphenolic compounds, has a greater ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) radical-scavenging capacity and more radioprotective effects than rutin, DMSO or vitamin C [103]. The stable free radical diphenyl picrylhydrazyl (DPPH) is used for the *in vitro* evaluation of free radical scavenging. The free-radical-scavenging abilities and radioprotective effects of flavonoids do not necessarily seem to be linked. Swertosin, a flavonoid that lacks *O*-dihydroxy groups in ring B but has c-glycosylation and a 3-hydroxyl group in ring C, had the lowest antioxidant activity *in vitro* by the DPPH method but had greater radioprotective effects in human culture lymphocyte than other compounds with higher free-radical-scavenging abilities [96]. Interestingly, flavonoids can pass through the plasma membrane to chelate and remove intercellular redox-active iron. This inhibits the H_2O_2-induced oxidative process mediated by iron [104] and protects against DNA damage induced by oxidative stress. This mechanism might contribute to the different antioxidant activities of flavonoids in *in vitro* assays such as the DPPH method and in cell culture.

Endogenous enzymes

Because IR induces a reduction in the level of cellular enzymatic antioxidants, the increase in the antioxidant status during flavonoid pretreatment of cells might further decrease the attack of free radicals on biomolecules such as DNA and membrane lipids, thereby decreasing the deleterious effects of radiation on cells and tissues. Administration of grape seed extracts rich in proanthocyanidins attenuated radiation-induced oxidative stress in mouse tissue by statistically significant increasing the activity of SOD, catalase and GPx [105]. Likewise, administration of quercetin, hesperidin and morin before the IR resulted in an increase in non-enzymatic and enzymatic antioxidant status, indicating that these polyphenolic pretreatments restored the endogenous antioxidant capacity to near normal levels [90,99,106].

SOD contains metal cofactors such as copper and zinc. Some ligand–metal complexes can mimic SOD activity and have radioprotective effects [29]. Although it is established that flavonoid–metal complexes can cause DNA damage through the prooxidative process, flavonoid–metal complexes can also scavenge superoxide radicals [107]. The flavonoid–copper complexes studied mimicked SOD activity and scavenged superoxide radicals produced by nitro blue tetrazolium *in vitro* more effectively than the flavonoid–iron complex [108]. There is little known about this SOD-mimicking activity, and more biological studies are needed to elucidate the exact mechanism by which this occurs.

Flavonoid–DNA interaction

The complex between histones and DNA results in condensation that protects mammalian DNA from strand breaks mostly induced

by the indirect, rather than the direct, effects of IR [34]. DNA strand breaks are increased by removing histones from chromatin because this gives free radicals increased access to DNA [34,109]. Replicating DNA results open chromatin structure with fewer attached histone proteins, which results in more DBS and DNA damage. The bisbenzimidazole derivative Hoechst 33342 [2′-(4-methoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-benzimidazole] and its analogs are DNA minor groove binding ligands that, when administered before IR, have protective effects on DNA lesions [110,111]. Hoechst compound contains a planar structure formed by its aromatic groups (two benzimidazole groups and one phenyl group), is positively charged and has an arc shape that 'fits' in the minor groove of DNA. The potent radioprotective effects of Hoechst compounds are attributable to this DNA–Hoechst interaction. A methylproamine analog of Hoechst had radioprotective effects approximately 100-fold greater than the classical aminothiol radioprotector, WR1065, possibly because of a stronger interaction with DNA through its positively charged amine group [112].

The interaction of flavonoids with DNA is one of the main mechanisms for protecting DNA from oxidative stress such as IR. Flavonoid intercalation into DNA, which has been established by spectroscopic and electrochemical methods [113–116], stabilizes the DNA structure and enables the flavonoid to react with nearby free radicals. Binding of flavonoids to DNA has been reported for quercetin, rutin, EGCG, morin and kaempferol. Free-energy calculations of binding thermodynamics revealed that binding occurs spontaneously with binding constants on the order of 10^3 M^{-1} and does not require energy [117]. Flavonoid intercalation also results in the deformation of the DNA double-helical structure [113]. Flavonoids bind to the major (adenine and guanine) and minor (thymine) grooves of DNA bases and to the backbone phosphate groups. Positively charged flavonoids such as delphinidin have a more stabilizing effect on the DNA duplex than neutral flavonoids such as quercetin or kaempferol owing to a stronger electrostatic interaction with the DNA backbone phosphates [118,119]. When this interaction was enhanced by the addition of a tertiary amine at position 7, binding was increased 1000-fold [117]. Flavonoids lacking positively charged side groups interact with the phosphate backbone of DNA primarily by hydrogen bonding through their hydroxyl groups [114,118,119]. The planar and aromatic moieties of flavonoids also contribute to DNA intercalation. The electronic properties of the flavonoid rings induce hydrophobic and aromatic π–π stacking interactions with DNA bases [74,113], and rings A and C are fused to provide a planar molecule that can intercalate between the stacked nucleic acid bases [117]. The planar benzopyran-4-one portion is probably localized and intercalated in the double-stranded DNA, more or less parallel to the adjacent planes of nitrogenous bases because of its hydrophobic nature. This hydrophobic force plays the most important part in intercalation of flavonoids into DNA [120,121]. By contrast, the coplanar catecholic group (ring B) is more likely to be oriented toward the external medium where it can form hydrogen bonds with phosphate groups in the DNA backbone [122]. The benzopyran-4-one portion of quercetin does not interact electrostatically with DNA because it has a higher affinity for positively charged structures at physiological pH, whereas DNA is mainly negatively charged [122,123]. The non-planar and hydrophilic flavonol, dihydroquercetin, which is saturated across the C2═C3 double bond, has less affinity for internal hydrophobic interactions with DNA. Naringin, a flavonoid without a C2═C3 double bond, does not interact with DNA alone but is capable of interacting with the nitrogenous bases in the presence of Cu(II), with which it forms a complex [124]. Hydroxyl groups were found to enhance DNA binding. Hydroxylation at the 7 position, as in 7-hydroxy flavone and 5,7-dihyroxy flavone, was identified as the most important for flavone–DNA interaction, whereas a methoxyl group at the 7 position reduced the binding constant [117].

There are several proposed mechanisms for protection against DNA damage conferred by flavonoids that are related to flavonoid–DNA interactions. First, intercalation of flavonoids into DNA double helices induces stabilization of DNA helical structure and condensation of DNA to a highly compact form that is less susceptible to attack by free radicals. Second, flavonoids have several hydroxyl groups, which easily donate hydrogen atoms to radicals formed in the DNA bases, as the flavonoid–DNA complex enables the flavonoid to repair the radical base very efficiently. Third, flavonoids can interact with the phosphate moiety of the DNA backbone through hydrogen bonding. The repair of sugar radicals is attributed to hydrogen donation from flavonoids through this bonding. Fourth, flavonoids act as reducing agents owing to their low oxidation potential and react easily with electron-accepting radicals before the ROS can attack DNA components.

Complexes between flavonoids and metal ions intercalated into DNA with a higher affinity than flavonoid alone, resulting in more DNA cleavage. This type of DNA damage was observed with complexes formed between flavonoids and copper, manganese or zinc, as previously discussed [125–127].

Induction of hypoxia

The induction of cellular hypoxia is related to the consumption of local oxygen. The production of ROS is decreased under hypoxia. This mechanism contributes to the protective effects of compounds against oxidative stress [128,129]. Several studies reported that flavonoids can induce expression of hypoxia-induced transcription factor-1 (HIF-1), a protein produced under hypoxia. HIF-1 is a key mediator of the cellular response to tissue hypoxia and acts to promote tissue survival at low oxygen levels [130]. The induction of HIF-1 expression results in reduced ROS [131] and delays the progression of cells past the G1 phase of the cell cycle, causing cell cycle arrest and leading to the inhibition of cell growth [130,131]. There is also a relationship between HIF-1 and iron because iron has a major role in the regulation of HIF-1. Flavonoids chelate cellular iron, resulting in the depletion of intracellular iron, and might be responsible for the reduction in ROS in response to iron-mediated oxidation [130]. Although there is no clear mechanism for the induction of HIF-1 expression by flavonoids, in quercetin and myricetin the catechol moieties were largely responsible for the observed induction [131]. More experiments in this field are required to determine whether HIF-1 induction is related directly to hypoxia or to the iron-chelating effect of flavonoids. The induction of HIF-1 and the hypoxia-response pathway by flavonoids results in a reduction of ROS levels, by which flavonoids confer their protective effects against genotoxicity induced by ROS.

Concluding remarks

IR produces ROS that attack and damage several targets, most crucially DNA. DNA damage results in genomic instability and mutation and/or cell death if the damage is not properly repaired. Flavonoids, by virtue of their chemical structure, protect against genomic instability induced by ROS. These compounds have prooxidant activity and can cause DNA cleavage *in vitro* that is increased in the presence of copper. Although there is no research to suggest that this deleterious effect of flavonoids occurs *in vivo* in normal cells, co-exposure of cancer cells to IR and to flavonoids results in enhanced radiation damage, with flavonoids acting as radiosensitizers. This effect might be beneficial to patients undergoing radiotherapy. Several *in vivo* and *in vitro* studies have shown flavonoids to have protective effects against IR-induced genotoxicity. There are several mechanisms for the mitigation of genomic instability induced by IR, including free radical scavenging, increased expression of endogenous enzymes, stabilization of the DNA double helix into a form less susceptible to damage and reduction of ROS levels by the induction of hypoxia. These effects of flavonoids are completely dependent on their structure, especially the number and position of hydroxyl groups and the C2═C3 double bond. Because flavonoids have a unique structure, these bioactive molecules have great potential as lead compounds for the design of new, more active compounds for ROS scavenging and DNA intercalation. These pharmacological properties might be useful in cancer treatment as novel anticancer or radio- and chemoprotective agents for patients during radiotherapy.

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