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Commentary

The flavonoid quercetin in disease prevention and therapy: Facts and fancies

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ABSTRACT

Biochemical and genetic studies on cellular and animal models on the mechanism(s) of action of phytochemicals provide a functional explanation of how and why a diet rich in fruits and vegetables is considered healthy. It is not unusual to find molecules that protect against diseases, which greatly differ from a physiopathological point of view, such as cancer and cardiovascular disorders. Quercetin falls into this category and possesses a broad range of biological properties. Uptake, metabolism and circulating concentrations of quercetin and its metabolites suggest that a regular diet provides amounts of quercetin (<1 μM) not compatible with its chemopreventive and/or cardioprotective effects. However, it appears relatively easy to increase total quercetin concentrations in plasma (>10 μM) by supplementation with quercetin-enriched foods or supplements. Multiple lines of experimental evidence suggest a positive association between quercetin intake and improved outcomes of inflammatory cardiovascular risk. The ameliorating effect of quercetin administration can be extended to other chronic inflammatory disorders but only if supplementation occurs in patients. Quercetin can be considered the prototype of a naturally-occurring chemopreventive agent because of its key roles in triggering the "hallmarks of cancer". However, several critical points must be taken into account when considering the potential therapeutic use of this molecule: (1) pharmacological versus nutraceutical doses applied, (2) specificity of its mechanism of action compared to other phytochemicals, and (3) identification of "direct" cellular targets. The design of specific clinical trials is extremely warranted to depict possible applications of quercetin in adjuvant cancer therapy.

1. Introduction

The last decade has seen the proliferation of an enormous number of scientific studies focused on the activity of non-nutritional compounds present in the diet and able to prevent the occurrence of degenerative diseases, such as cancer and cardiovascular pathologies. This heterogeneous class of molecules, generally known as phytochemicals, includes vitamins (carotenoids) and food polyphenols, such as flavonoids, phytoalexins, phenolic acids, indoles and sulfur-rich compounds [1,2]. They are widely present in fruits, vegetables, and beverages (tea, wine, beer) and in many dietary supplements and herbal remedies. However, what largely attracts scientists' interest is the number of compounds available for testing, with more than 10,000 phytochemicals potentially present in nature. The wide range of biological activities remains uncharacterized for most compounds [3]. Phytochemicals, in fact, trigger cellular pathways that lead to the prevention and/or amelioration of pathological conditions associated with cancers, and cardiovascular and neurodegenerative diseases [4,5]. Although this positive association is debated and is the subject of criticism [6], biochemical and genetic studies on cellular and animal models on the mechanism(s) of action of phytochemicals provide a functional explanation of how and why a diet rich in fruits and vegetables can protect against degenerative diseases [6,7].

According to these epidemiological data, it is not unusual to find molecules that exhibit protective effects against diseases that greatly differ from a physiopathological point of view. This is the case, for example, for resveratrol, a cancer-preventing agent also possessing cardio-protective properties and, more recently, with potential anti-aging effects [1]. Similarly, sulforaphane was originally studied as a chemopreventive agent [8] before the description of its efficacy in preventing neurodegeneration [9].

The present review focuses on quercetin, which represents the most abundant dietary flavonoid found in a broad range of fruits, vegetables and beverages, whose antioxidant and anti-inflammatory properties have been associated with the prevention and therapy of cardiovascular diseases and cancer. We selected and analyzed key aspects of quercetin's effects in order to propose a unified model of its mechanism of action. In doing this, we apologize in advance for the many citations omitted due to space limitation and hope that this commentary may help to predict future developments in the field.

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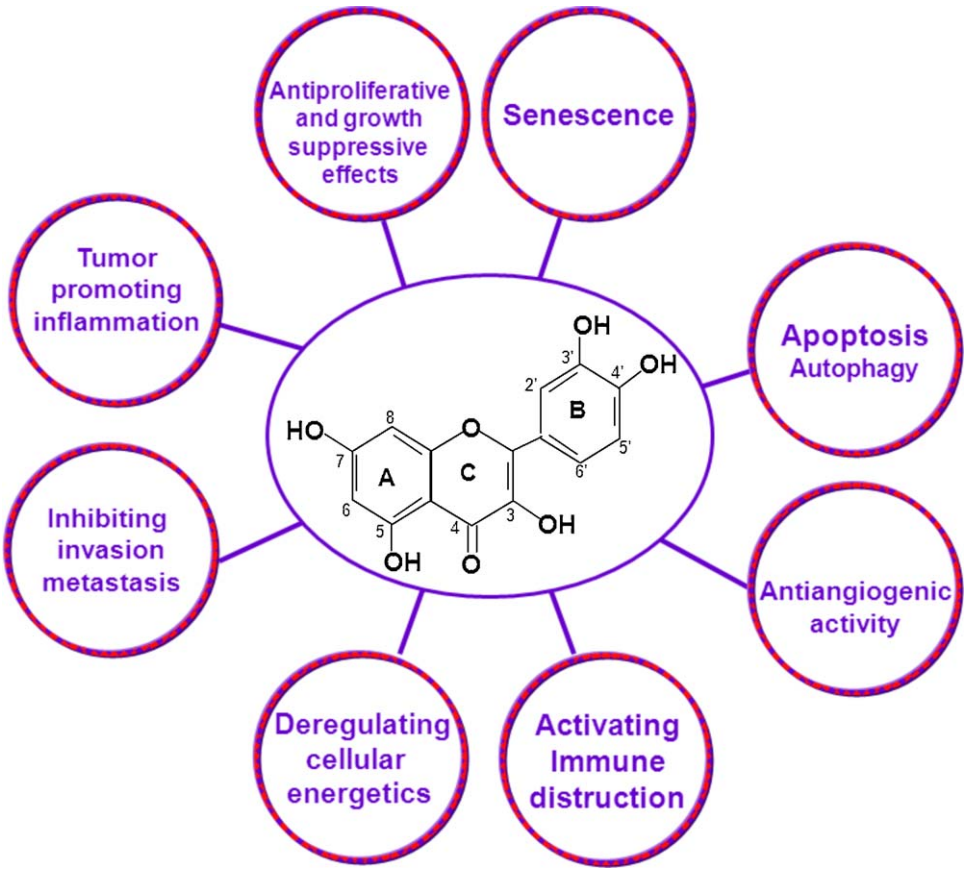

Fig. 1. The contribution of quercetin to different "hallmarks of cancer", according to Hanahan and Weinberg ([34,35]) (see text for details).

2. Quercetin absorption, metabolism and bioavailability

One of the reasons for the success of quercetin (3,3′,4′,5,7-pentahydroxyflavone; Fig. 1), as described in the literature (at the moment, more than 8000 citations in PubMed[2]), is probably due to the relatively high bioavailability of the molecule compared to other phytochemicals. The daily intake of quercetin in the diet has been estimated as 5–40 mg/day [10] although these levels can increase up to 200–500 mg/day in individuals who consume high quantities of fruits and vegetables rich in flavonols (apples, onions, tomatoes) [11]. The quercetin in foods is not present as aglycon (*i.e.*, without sugar groups), but it is differently glycosylated. Therefore, its bioavailability depends on the type of glycosides present in different food sources. Despite the original belief that only the free form of quercetin could be absorbed at the intestinal level by passive diffusion due to its hydrophobic nature, later studies have surprisingly demonstrated that the adsorption of quercetin glycosides almost doubles that of its corresponding aglycon [12]. The biochemical explanation for the higher bioavailability of quercetin glycosides probably resides in either deglycosylation processes at the intestinal level and/or carrier-mediated transport [13,14]. After absorption, quercetin is metabolized in different organs, such as the small intestines, colon, liver and kidney. Here, the molecule is conjugated to methyl and sulfate groups and glucuronic acid to generate its major conjugates in humans: 3′-*O*-methylquercetin (isorhamnetin), quercetin-3-*O*-glucuronide, 3′-*O*-methylquercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate [11]. It is worthwhile to report that, according to some authors, neither glycosides of quercetin nor free aglycone is present in plasma. Earlier detection of these species was probably due to experimental artifacts [15]. In addition, the absorption of quercetin is also influenced by gut microflora, which, in rats, converts more than 95% of the [2-^{14}C]quercetin-4′-glucoside to phenolic acids [16] while, in humans, half of the quercetin-3-rutinoside is probably metabolized to phenyl-C_2 acids by colonic microflora [17]. As a result of its absorption and metabolism, total quercetin derived from the diet is present in plasma at the nanomolar range (<100 nM) but can be increased to micromolar concentrations after supplementation. As an example, 28 days of supplementation with 1 g/day of quercetin increased plasma concentrations to 1.5 μM [15,18]. Inter-individual variability can justify the broad range of determinations published after quercetin supplementation in healthy volunteers. These variations can be explained by evoking the different bioavailability of quercetin glycosides present in different foods and the polymorphism of intestinal enzymes in humans and animal models [15].

In view of the potential clinical use of the molecule, quercetin's half-life and tissue distribution provide useful information. The half-lives of the molecule and its metabolites range between 11 and 28 h, which suggest the possibility of significantly increasing plasma concentrations upon continuous supplementation [13,15]. Quercetin and quercetin metabolites are widely distributed in rat tissues with the highest concentrations in lung (3.98 and 15.3 nmol/g tissue for a 0.1% and 1% quercetin diet, respectively). In pigs, higher concentrations have been detected in the liver (5.87 nmol/g tissue) and kidneys (2.51 nmol/g tissue) [19].

Recent efforts have been devoted to identifying novel strategies to ameliorate quercetin bioavailability. Table 1 reports a selection of these approaches with a description of the most significant improvements obtained in terms of increased quercetin uptake in cellular and animal models.

Data on quercetin efflux and cellular resistance mediated by P-glycoprotein are fragmentary and contradictory because quercetin,

[2] http://www.ncbi.nlm.nih.gov/pubmed.

Table 1
Selection of different approaches to increase uptake and bioavailability of quercetin.

Compound[a]	Chemical modification	Effects	Reference
QC12	Quercetin-glycin-carbammate conjugate	QC12 is not orally bioavailable. After i.v. administration, plasma concentration of QC12 was 108.7 ± 41.67 μM.	[82]
QE	Quercetin-glutamic acid conjugated	Increased water solubility, stability, and cell permeability. Higher half-life respect to QC12	[83]
Liposomal Q	Quercetin encapsulated in polyethylene glycol 4000 liposomes	Liposomal Q accumulates in tumour tissues and induces apoptosis *in vivo* and *ex vivo*. Half-life in plasma 2 h	[84]
Q nanoparticles	Quercetin encapsulated on poly-D,L-lactide (PLA) nanoparticles	Controlled release from PLA nanoparticles. Q nanoparticles have the same antioxidant activity as control Q	[85]
Q nanocrystals	Quercetin nanocrystals fabricated using high-pressure homogenization	Higher solubility. The antioxidant activity and reducing power of the Q nanocrystals were more effective than control Q	[86]
Q derivatives	3′,4′,5′-trimethoxyflavonol	In APC^{min+} mice and HCT-116 injected nude mice, decreased tumour development, increased apoptosis and 1.5-3 fold increase in p53 expression	[87]
Q derivatives	3-(4-O-triphenylphosphoniumbutyl) Q iodide (Q3BTPI) and its tetracetylated analogue (QTA3B)	Increased permeability in isolated rat mitochondria	[88]
Q micelles	Q in polymeric micelles of PEG-OCL (poly(ethylene glycol)-b-oligo(ε-capro-lactone)) with naphthyl or benzyl end groups	Increased solubility of Q entrapped in mPEG750-b-OCL micelles (up to 1 mg/ml; approx. 110 times higher than that of Q in water)	[89]
Kollidon® 25 (PVP K25)	Solid dispersions of Q with PVP K25	Q solubility was increased by PVP K25 in concentration dependent manner. Improved solubility even 436 higher than pure Q	[90]

[a] Q indicated quercetin aglycone.

similar to other phytochemicals, can both increase or inhibit P-glycoprotein-mediated efflux [20]. In malignant cells, many reports indicate that a combined treatment with quercetin and different chemotherapeutic agents ameliorates therapy efficacy. Several factors such as drug interaction, dose, frequency and timing of quercetin intake probably influence the role of quercetin in the multidrug resistance phenomenon.

3. Quercetin toxicity and safety

Earlier studies in the 1970s recognized quercetin as genotoxic by standard tests (reviewed in [11]). However, quercetin's *in vitro* mutagenicity was not confirmed by *in vivo* tests in animal models, where the molecule failed to induce any significant changes when mutagenicity/genotoxicity endpoints in somatic cells were determined [11]. In 1999, IARC (the International Agency for Research on Cancer) concluded that quercetin is not classifiable as carcinogenic to humans, which is in agreement with the daily intake of the molecule in the diet and the absence of revealed cases of adverse effects for human health [21]. During a 2-year study conducted by NTP (National Toxicology Program), male F344/N rats fed 2 g/kg body weight/day of quercetin (corresponding to a dose of 140 g for a 70 kg individual) showed severe chronic nephropathy, hyperplasia and neoplasia of the renal tubular epithelium. At lower doses, from 50 to 500 mg/kg/day, no significant adverse effects were reported. However, parallel studies performed using the same rat model failed to confirm the renal histopathological effects of quercetin (Table 2 in [11]). In humans, the unique phase I clinical trial of quercetin currently recommends a dose of 1400 mg/m^2, which corresponds to about 2.5 g for a 70 kg individual, administered via intravenous infusion at 3-week or weekly intervals [22]. At higher doses, up to 50 mg/kg (about 3.5 g/70 kg), renal toxicity was detected without signs of nephritis or obstructive uropathy. Human studies have failed to show any adverse effects associated with the oral administration of quercetin in a single dose of up to 4 g or after one month of 500 mg twice daily (reviewed in [23]).

Table 2
Cellular substrates directly targeted by quercetin.

Targets	Binding site	Concentration	Cellular effects	Reference
MEK1	Activation loop	1–2 μM	Apoptosis/Cell cycle/Growth arrest	[46]
PI3Kγ	ATP-binding site	3.8 μM	Apoptosis/Cell cycle/Growth arrest	[60]
IKK α/β	ATP and IκBα binding sites	IC50 11 μM (α) IC50 4 μM (β)	Apoptosis Inflammation	[91]
Telomerase	n.d.	10 μM	Apoptosis/Senescence	[92]
Hck (Src tyr kinase family)	ATP-binding site	2 μM	Apoptosis/Cell cycle/Growth arrest	[44]
IRE1-RNAse	Ligand binding pocket at the dimer interface	25 μM	Autophagy	[69]
Wnt/β-Catenin	Binding of β-catenin to Tcf-4	10–20 μM	Apoptosis	[66]
CK2	ATP-binding site. Competitive inhibitor	IC50 = 0.92 μM/K_i = 1.18 μM	Apoptosis	[93]

n.d., not determined.

Despite this reassuring information on quercetin's safety, the toxicity of the molecule may paradoxically arise from its well-known antioxidant properties, which are a function of its chemical structure and particularly the presence and location of its hydroxyl (–OH) substitutions and its catechol-type B-ring (Fig. 1). Within flavonoids, quercetin is the most potent scavenger of ROS (reactive oxygen species) and RNS (reactive nitrogen species) *in vitro*, and its contribution to the total plasma antioxidant capacity is 6.24 times higher than trolox, which has been used as an antioxidant reference [13]. However, when employed as an antioxidant, quercetin becomes oxidized to generate quercetin–quinone (QQ) with its tautomeric forms. QQ, like other semiquinone radicals and quinones, is toxic because of its ability to arylate protein thiols. Protection against QQ may arise from GSH, the most abundant endogenous thiol, with the transient formation of adducts called GSQ, which possess a very short half-life and are rapidly dissociated into GSH and QQ. This implies that in the presence of a low concentration of GSH QQ trapping may not be efficient, and the quercetin quinones may become free to react with other thiol groups, *e.g.*, protein sulfhydryls [13]. However, potential toxic effects of QQ species have not yet been proven *in vivo*.

4. Quercetin and cardiovascular diseases

The role of quercetin in preventing cardiovascular diseases has been largely associated with its anti-inflammatory and antioxidant properties. Although supplementation with quercetin (1 g/day for 28 days) in healthy subjects had no effect on total serum LDL, HDL cholesterol and triglyceride levels [23], earlier studies have linked regular consumption of flavonoids in foods with a reduced risk of death from coronary heart disease [10,24]. No alteration of other thrombogenic risk factors, including platelet aggregation, platelet thromboxane B2 production, blood pressure or resting heart rate, were observed. In these subjects, quercetin intake was approximately 50-fold greater than the dietary intake associated with lower coronary heart disease mortality on the basis of epidemiologic studies. In fact, plasma quercetin concentrations increased by approximately 23-fold compared to control subjects [18]. Similarly, two different preparations containing quercetin, vitamin C, and niacin (500 mg quercetin, 125 mg vitamin C, and 5 mg niacin, or 1000 mg quercetin, 250 mg vitamin C, and 10 mg niacin) supplemented for 12 weeks in a large population of adults (n = 1002; 60% women), which varied widely in age and body mass indices, had a negligible influence on cardiovascular risk factors with a very modest decrease in mean arterial blood pressure and inflammatory markers, *e.g.*, IL-6 (interleukin-6). Both treatments showed an increased concentration of circulating quercetin. In these studies, the lack of an effect due to the molecule can be attributed to the physical status of the enrolled subjects, who were healthy and probably did not require extra antioxidant supplementation. In fact, more recent studies performed on cardiac patients or populations at cardiovascular risk indicated a protective effect with quercetin supplementation. In stage 1 hypertensive patients, 730 mg quercetin/day for 28 days was associated with reduced systolic, diastolic and mean arterial pressures [25]. Similarly, in an at-risk population of 93 overweight or obese subjects with metabolic syndrome traits, supplementation with 150 mg quercetin/day for 6 weeks decreased systolic blood pressure and plasma concentrations of atherogenic-oxidized LDL but did not affect TNF (tumor necrosis factor)-α and C-reactive protein (CRP) when compared to the placebo, and mean fasting plasma quercetin concentrations increased from 71 to 269 nM [26].

The cardioprotective activity of quercetin in patients is enforced and confirmed by studies performed on cellular and animal models, which also suggest a potential mechanism of action for the molecule. In fact, quercetin reduced inflammation in isolated human macrophages and adipocytes. Treatment of macrophages with quercetin attenuated the basal expression of inflammatory genes, such as TNF-α, IL-6, IL-8, IL-1β, interferon-γ-inducible protein-10, and cyclooxygenase(COX)-2, a marker of prostaglandin production [27]. In primary human adipocytes, quercetin ameliorated several events dependent on TNF-α, such as the expression of inflammatory genes; the secretion of IL-6, IL-8, and MCP-1 (monocyte chemoattractant protein-1); and TNF-α-induced NF-κB transcriptional activity [28]. The molecule also prevented the TNF-α-mediated serine phosphorylation of insulin receptor substrate-1 and protein tyrosine phosphatase-1B gene expression and suppressed insulin-stimulated glucose uptake [28]. In this context, it is worthwhile to note that quercetin potentiated glucose and glibenclamide-induced insulin secretion and protected β-cells against oxidative damage with ERK1/2 playing a major role in those effects [29]. In animal models, orally administered quercetin (10 mg/kg) as a pretreatment for Wistar rats was provided daily for 7 days and protected the rats from experimentally-induced myocardial infarction by subcutaneous injection of isoproterenol by lowering ST-segment elevation and decreasing levels of lipid peroxidation products in the plasma and heart [30]. Quercetin pretreatment also significantly reduced levels of total cholesterol, triglycerides and free fatty acids in serum, heart and heart mitochondria and serum phospholipids, as well as reduced levels of serum LDL and very LDL cholesterol and significantly increased serum HDL [30]. A recent study reported on the antioxidant and anti-inflammatory properties of quercetin in two humanized models of cardiovascular disease [31]. When quercetin (0.1%; w/w in diet) was given to human CRP transgenic mice, a humanized inflammation model, and ApoE*3Leiden transgenic mice, a humanized atherosclerosis model, quercetin quenched IL-1β-induced CRP expression in the former and reduced atherosclerosis (40%) in the latter by significantly lowering the circulating inflammatory risk factors SAA (serum amyloid A proteins) and fibrinogen. It is worthwhile to note that, in both cases, the quercetin plasma concentration ranged between 13 and 19 μM, which are values comparable to those measured in rodents treated with the same doses (0.1%, w/w) [31]. In cultured human endothelial cells, quercetin protected against H_2O_2-induced lipid peroxidation and reduced the transcriptional activity of NFκB in human hepatocytes [31]. Finally, to support the cardioprotective effects of oral quercetin supplementation, several authors have described that its metabolites, such as isorhamnetin (3-methyl-quercetin) and quercetin 3-*O*-β-D-glucuronide (Q3GA), show preventative effects against arteriosclerosis. Isorhamnetin produces endothelium-independent vasodilator effects in animal vascular tissues, such as rat aorta, mesenteric arteries, portal vein and porcine coronary arteries [32], while Q3GA inhibits platelet-derived growth factor-induced cell migration and proliferation in VSMCs (vascular smooth muscle cell) and attenuated angiotensin II-induced VSMC hypertrophy [33].

5. Quercetin and cancer

The vegetable kingdom is an almost inexhaustible source of phytochemicals with potential chemotherapeutic and chemopreventive activities [2]. Quercetin can be considered the prototype of a naturally-occurring chemopreventive agent because its described biological activities (antioxidant, anti-inflammatory, anti-proliferative, pro-apoptotic and anti-angiogenic) span through all stages of carcinogenesis from initiation to invasion and metastasis and act on different genetic, biochemical and immunological aspects that underpin the development and maintenance of tumors. According to a pivotal study by Hanahan and Weinberg published in 2000 [34] and recently reviewed [35],

six "hallmarks of cancer" have been defined. The acquisition of these properties, which in turn reflect changes in the biochemical pathways of signal transduction (caused by the activation of oncogenes and disabling tumor suppressor genes), are made possible by two "enabling" characteristics: genomic instability generating random mutations and the chronic inflammatory state driven by the immune system [34,35]. The ability of quercetin to interfere with different targets identified as "hallmarks of cancer" makes this molecule, together with several other phytochemicals, a multi-target inhibitor with pleiotropic and synergistic effects in tumor cells (Fig. 1) [5]. An abundant literature (see below for detailed description) suggests that quercetin can be efficient at treating cancer by inducing cell death or cell cycle arrest preferentially in cancer cells versus their normal counterparts through a process involving the down-regulation of selective oncogenes (such as Mcl-1, Ras, MEK, PI_3K), or the up-regulation of tumor suppressor genes (p53, p21), which, in turn, enhance selective pathways leading to the elimination of cancer cells (Fig. 1).

5.1. Antiproliferative and growth-suppressing effects

The proliferation of normal cells is highly regulated. Growth factors bind and recruit transmembrane receptors that activate signaling pathways, which regulate progression through the cell cycle, cell growth, cell survival and energy metabolism [34,35]. Deregulation of these processes, or defects in one or more of these steps controlling proliferation, can turn a normal cell malignant. The role of quercetin in cell proliferation and survival is widely documented in the literature [13,23]. Quercetin inhibits the growth and proliferation of cancer cell lines of different origins (prostate, cervical, lung, breast, and colon) *in vitro*. In many *in vivo* studies where animal models are used, cancer is induced by a high dose of carcinogens, a condition very different from the carcinogenesis process occurring in humans. For example, feeding dietary amounts of quercetin results in the inhibition of intestinal crypt cell proliferation and the suppression of aberrant crypt formation [36]. Several mechanisms have been proposed to explain the effects of quercetin on cell growth, and a large number of these studies focus on the ability of this compound to target specific regulatory proteins, including cyclins (cyclin A, B, D or E), cyclin-dependent kinases (Cdks) and CDK inhibitors, such as $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ [37]. In a recent study, low concentrations of quercetin (2 μM) decreased the activity of 16 cell cycle-related kinases by more than 80%, including ABL1, Aurora-A, -B, -C, CLK1, FLT3, JAK3, MET, NEK4, NEK9, PAK3, PIM1, RET, FGF-R2, PDGF-Rα and Rss [38]. Quercetin causes cell cycle arrests at the G2/M transition or G1 phase in different cell types. In OCM-1 (melanoma cells), quercetin at a high concentration (70 μM) blocked cell proliferation and arrested cells at the G1 phase by inhibiting Cdk2 activity and up-regulating $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ [37]. In MCF-7 (a human breast carcinoma cell line), quercetin inhibited cell cycle progression through transient M phase accumulation and subsequent G2 arrest. This mechanism involves the inhibition of cyclin B1-associated Cdc2/Cdk1 kinase activity, which decreases the expression of cyclin B1 and increases $p21^{CIP1/WAF1}$ protein synthesis [39]. Relatively low doses of quercetin (1–10 μM) administered during a 24-h interval to breast cancer cells mimic the conditions of daily consumption and induce cell cycle arrest at the G0/G1 phase. Quercetin inhibits cyclin-Cdks activity through the induction of $p21^{CIP1/WAF1}$ with a concomitant decrease of phosphorylated Rb [40]. Interestingly, the proliferation of the MCF-10A cell line, which resembles a normal breast epithelium, was not affected by 10 μM quercetin, which suggests that the molecule has a cancer-specific antiproliferative effect.

Quercetin is a potent inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase activity, which plays a key role in cell proliferation [41,42]. The double bond between the C2 and C3 in the C ring and the OH groups of the C3′ and C4′ in the B ring of the molecule (Fig. 1) are critical for accessing the kinase's binding site, which is placed at, or near to, the ATP fold of protein kinases [43]. However, as for Cdks, quercetin does not directly inhibit EGFR but interferes with different signaling pathways downstream of EGFR that regulate cell proliferation and survival (Table 2). One of these signals could be the Src family tyrosine kinase Hck, which is expressed in lymphoid and myeloid cells and involved in B-cell receptor signaling. Crystallographic studies have demonstrated that the inhibition of the enzymatic activity of Hck is a consequence of intramolecular interactions between the enzyme's Src-homology domains SH2 and SH3 with a concomitant displacement of the elements present in the catalytic domain. Quercetin has been employed to improve the crystallographic resolution of Hck because of its ability to interact with key residues in the catalytic domain [44].

Pharmacologically safe doses of quercetin (25 μM) inhibit the phosphoinositide-3 kinase (PI_3K)-AKT/PKB pathway in PTEN-null cancer cells (where the AKT/PKB pathway is constitutively activated) [45]. Raf and MEK protein kinases are also direct molecular targets of quercetin, which decrease MEK1 activity more efficiently than PD098059, a specific MEK inhibitor [46]. Docking data suggest that quercetin forms a hydrogen bond with the backbone amide group of Ser^{212} in MEK1, which represents the key interaction for stabilizing the inactive conformation of the activation loop of MEK1 [46]. Transcriptomic and proteomic changes occurring in the distal colon mucosa of rats supplemented with 10 g of quercetin/kg diet for 11 weeks indicated that quercetin significantly down-regulated the potentially oncogenic MAPK *in vivo* [47].

5.2. Senescence induction and telomerase inhibition

Cellular senescence is a program of irreversible cell cycle arrest that normal cells undergo in response to progressive shortening of telomeres, changes in telomeric structure, oncogene activation or oxidative stress [35]. Senescence induction by phytochemicals could be a new and alternative strategy of chemoprevention in addition to apoptosis or autophagy in tumor cells resistant to these tumor-suppressing pathways. In a recent paper, quercetin and resveratrol (at low doses) cooperated to induce a senescence-like growth arrest in a very resistant glioma cell line [48]. Even if the exact molecular target was not identified, the authors showed that the combined treatment caused a reduction in AKT phosphorylation. Quercetin also targeted telomerase in senescence induction and in evading replicative immortality (Table 2). Telomerases are specialized DNA polymerases that add repeating telomere segments to the ends of DNA. These enzymes are expressed at functionally significant levels in almost 90% of immortalized cells, including human cancer cells. The presence of a high telomerase activity is correlated to both senescence and apoptosis resistance [35]. In fact, quercetin and other polyphenols (*e.g.*, epigallocatechin gallate; EGCG) inhibit telomerase activity and display a low IC_{50} (0.45–4.5 μM, depending on the incubation buffer) in an *in vitro* cell-free system. This effect was recently confirmed in colon adenocarcinoma (Caco-2) and breast adenocarcinoma (MCF-7) cell lines where quercetin was effective as a telomerase inhibitor at 25 μM [49].

5.3. Cell death induction

Apoptosis, or programmed cell death, is one of the most important mechanisms (in addition to senescence and macro-autophagy) activated by pre-malignant cells to arrest the multistep process of carcinogenesis, which, in many cases, generates cancer

cells that are resistant to the induction of cell death [35]. Apoptosis can be modulated by both extrinsic and intrinsic pathways. The extrinsic pathway is regulated by cytokines (mainly CD95L and TRAIL), which bind to members of the superfamily of tumor necrosis factor receptors (TNF-R). These cytokines, which are mainly secreted by cytotoxic T cells or natural killer (NK) cells, are important in preventing the growth of tumors by the immune system, especially in the early stages, when these cells are involved in the recognition and subsequent elimination of transformed cells (immunosurveillance) [50]. CD95L and TRAIL ligands (death ligands; DL) recognize their specific receptors present on tumor cells (CD95, TRAIL-R1/R2), called death receptors (DR), and trigger a cascade of apoptotic signals culminating in the activation of a class of cysteine proteases (caspases) that lead to the destruction and elimination of tumor cells without inflammation and tissue damage [51]. However, the mechanisms of acquired DR resistance in tumors responding to CD95 and TRAIL stimulation remain largely unknown. Paradoxically and in specific situations, CD95L and TRAIL, similar to TNF-α, are likely to have anti-apoptotic functions in addition to their well-known apoptogenic features. For example, it is known that DL are able to stimulate pro-survival pathways controlled by PI_3K/AKT and MAPK and activate inflammatory nuclear factor-κB (NF-κB)-dependent responses in different cell lines [51].

The apoptotic intrinsic pathways, which are controlled at the mitochondrial level, are activated by genotoxic damage, which prevents cells from replicating abnormally. In this case, Bcl-2 family members play a key role including factors possessing pro-apoptotic (Bax, Bak, PUMA, NOXA, Bim, Bid) or anti-apoptotic activities (Bcl-2, Mcl-1, Bcl-xL, Bfl-1/A1) [52].

Quercetin is able to bypass DR resistance through multiple mechanisms. A good example is represented by works in a human acute lymphocytic leukemia cell line (HPB-ALL) where quercetin, administered at non-cytotoxic concentrations, induces apoptosis only when present in combination with a CD95 agonistic antibody [53]. At the molecular level, quercetin lowered intracellular ROS, the reduced mitochondrial transmembrane potential and, thereby, left the expression of the CD95 receptor unchanged. However, the ability of quercetin to sensitize HPB-ALL cells to CD95-induced apoptosis was not due to its antioxidant properties because other dietary flavonols structurally and functionally related to quercetin (*e.g.*, catechin, myricetin) did not mimic quercetin apoptogenic activity. However, these analogs did maintain their antioxidant capacity [54]. This finding has been confirmed in other cellular models for human leukemia of lymphoid (Jurkat) or myeloid (U-937, K-562) origin and also extended to cell lines resistant to TRAIL-induced apoptosis [55]. It is worthwhile to mention that the same concentrations applied to leukemia cell lines did not induce apoptosis in lymphocytes isolated from the peripheral blood of healthy subjects and in the same cell lines (U-937, K-562) induced to differentiate [55]. These observations have been confirmed in a different study on prostate cancer cell lines where 5–10-fold higher quercetin concentrations were used [56]. In cell lines resistant to DR-induced apoptosis, quercetin activity requires factors present at the DISC (death-inducing signaling complex) level, as shown by the activation of apical caspases (caspase-8) [54,55].

In an *ex vivo* model of chronic lymphocytic leukemia (CLL), quercetin, at low doses (10–20 μM), was able to sensitize B-cells isolated from patients to DR-induced apoptosis and to fludarabine, a first-line chemotherapeutic drug in CLL [57]. The anticancer and pro-apoptotic effect of quercetin has also been demonstrated *in vivo* using a mouse model of pancreatic cancer [58]. Here, quercetin induced apoptosis and prevented metastasis. In addition, if the molecule was associated with other phytochemicals (resveratrol), a synergistic effect was measured by the activation of caspase-3 and inhibition of NF-κB [58]. In a more recent *in vivo* study, quercetin acted synergistically with sulforaphane to inhibit the growth of pancreatic cancer stem cell- enriched xenografts, to reduce the proliferation, angiogenesis, and expression of cancer stem cells markers and to induce apoptosis without toxicity to normal cells or mice [59].

From a molecular point of view, several mechanisms can be evoked to explain the ability of quercetin to bypass apoptotic resistance. Its direct targets are key anti-apoptotic protein kinases, such as, PI_3K [60] and MEK1 [46], that in turn, phosphorylate and inactivate BH3-only proteins like Bad or Bid and Bim, respectively [61]. Quercetin also binds and inhibits CK2 kinase[3] and CK2-dependent phosphorylation as a global mechanism for inhibiting caspase signaling [62]. Moreover, considering the limited oral bioavailability of quercetin and its rapid metabolism in cells, the report that its *O*-methylated and glucuronide metabolites were able to inhibit the AKT/PKB and ERK1/2 pathways and, thus, induce apoptosis [63] was particularly interesting. In U-937 leukemia cells and mice xenografts injected with this cell line, the pro-apoptotic effects of quercetin resulted in both the increased expression of the pro-apoptotic factor Bax and the inhibition of anti-apoptotic Mcl-1 [64]. The effect of quercetin on Mcl-1 expression has been recently confirmed and extended. Quercetin down-regulates Mcl-1, which is often up-regulated in CLL, and acts directly or indirectly on its mRNA stability and protein degradation, which suggests that this same mechanism may bypass resistance to apoptosis in leukemia cells isolated from CLL patients and sensitized B-cells to apoptosis induced by drugs and DR inducers [65]. These authors also demonstrated that quercetin caused apoptosis in both transformed and primary leukemia cells but not in normal blood peripheral mononuclear cells at concentrations up to 50 μM.

Finally, the dysregulation of the Wnt/β-catenin pathway plays a central role in early events in colorectal carcinogenesis as well as in ALL (acute lymphoblastic leukemia). Because quercetin is a potent Wnt pathway modulator, which inhibits the transcriptional activity of the β-catenin/Tcf complex to its specific DNA-binding sites [66], treatment with quercetin induces apoptosis in different ALL cell lines, which demonstrates that this pathway may represent an important therapeutic target in ALL [67].

These data demonstrate that quercetin may be considered in the treatment of leukemia because the molecule preferentially induces apoptosis in leukemia cells without damaging normal lymphocytes. This process involves Mcl-1 or Wnt/β-catenin pathway down-regulation (“oncogene addiction”), which, in turn, potentiates downstream pathways leading to apoptosis.

5.4. Autophagy induction

Autophagy is a cellular process by which different cytoplasmic components, including organelles, are targeted for degradation by autophagosomes. It is known that autophagy induced by “oncogenic stress” protects against malignant transformation and triggers autophagy protein-dependent cell senescence or cell death. Human cancer cell lines bearing activating mutations in the *ras* oncogene commonly show high levels of basal autophagy. In addition, down-regulation of essential autophagy proteins impairs cell growth. In Ha-*ras* transformed colon cancer cells, quercetin induces autophagic cell death by down-regulating levels of oncogenic *ras* [68], but the exact molecular target is still unknown. Endoplasmic reticulum (ER) stress, which is basically activated in many cancers, results in autophagic cell death [69]. A recent study identified a novel molecular target of quercetin that induces ER stress, the kinase IRE1 (inositol-requirement-1)-RNase [69]. This protein contains a cytoplasmic portion possessing both kinase and

[3] Russo M et al., unpublished.

RNase domains, which are involved in the splicing and activation of the transcription factor XBP1. In turn, XPB1 modulates the expression of chaperones, which are key regulators of protein folding. In this study, the authors demonstrated that quercetin activates yeast IRE1 RNase and potentiates activation by ADP, a natural ligand that engages the IRE1 nucleotide-binding cleft. Enzyme kinetics and the structure of a co-crystal of IRE1 complexed with ADP and quercetin revealed the engagement of quercetin at an unanticipated ligand-binding pocket at the dimer interface of the IRE1 kinase extension nuclease (KEN) domain (Table 2).

5.5. Anti-angiogenic activity

Angiogenesis induction is essential for tumor sustainment, *i.e.*, to access nutrients and oxygen and evacuate metabolic wastes and carbon dioxide. It is characterized by the formation of new vessels from a pre-existing microvascular network. Generally, this physiologic process becomes quiescent and is only transiently activated. However, in tumor progression, it is usually activated, and the new vessels help sustain expanding neoplastic growth. The angiogenic switch is regulated by different factors that induce (vascular endothelial cell growth factor, VEGF, matrix metalloproteinase),or oppose (thrombospondin) angiogenesis [35]. Inhibition of angiogenesis is a promising therapeutic approach for controlling of tumor growth and progression. Quercetin inhibits several important steps of angiogenesis. Treatment with 25 μM of quercetin for 24 h inhibited tube formation of human microvascular dermal endothelial cells and human umbilical vein endothelial cells (HUVEC) [70]. The effects of quercetin and its main circulating conjugates, Q3′S (quercetin-3′-sulfate) and Q3G, were investigated *in vivo* using an angiogenesis process induced by VEGF. The authors showed that Q3G and quercetin itself had no effect on quiescent endothelium, while the same molecules inhibited endothelial functions and *in vivo* angiogenesis induced by VEGF. Inversely, Q3′S significantly increased the growth of quiescent endothelia and had no effect on cell proliferation stimulated by VEGF. These data indicate that the effects of circulating quercetin conjugates on angiogenesis are different depending on the nature of the conjugate [71].

5.6. Activation of immune destruction

Avoiding immune destruction is another hallmark of cancer and an emerging field in tumor therapy and prevention. In particular, deficiencies in the development or function of cytotoxic CD8+ lymphocytes (CTL), CD4+ helper T-cells or NK cells in mice models lead to an increased tumor incidence. Moreover, clinical epidemiology supports the existence of an antitumoral immune response in some types of human cancers [35]. Recently, it has been reported that quercetin was able to enhance susceptibility to NK cell-mediated lysis of cancer cells through the induction of NKG2 (natural killer group 2, member D) ligand. This ligand interacts with the NKG2D receptor on the NK surface and mediates the immune response by NK against tumors. It is well-known that UV and ionizing radiations, chemotherapeutic agents (5-fluorouracil and cisplatin) and histone deacetylase inhibitors (*e.g.*, valproic acid) are able to induce the expression of NKG2 ligands and DR5 in cancer cells, which make them susceptible to NK elimination. Using different human leukemia and adenocarcinoma cell lines (K-562, SNU1 and SNU-C4) treated with quercetin under different concentrations (from 10 to 100 μM) and incubation times (3–24 h), the authors demonstrated that the flavonoid could increase the expression of different NKG2D ligands at the transcriptional and cell surface protein levels. This effect may be due to the inhibition of PI_3K and NF-κB pathways and to a concomitant decreased expression of HSP70 [72].

5.7. Effects of quercetin on other hallmarks of cancers

Among the other "hallmarks of cancer", circumstantial evidence links quercetin to tumor-promoting inflammation, invasion and metastasis and deregulated cellular energetics (Fig. 1).

The COX-2-catalyzed synthesis of prostaglandin E2 plays a key role in inflammation and its associated diseases. In addition, pro-inflammatory cytokines are considered potential markers for colorectal carcinogenesis. It is known that quercetin and quercetin conjugates reduce COX-2 mRNA expression and activity in both unstimulated and interleukin-1β-stimulated Caco2 cells [73]. In rats receiving a diet containing quercetin (0–4.5 g/kg) and injected subcutaneously with azoxymethane, quercetin suppresses the formation of early preneoplastic lesions in colon carcinogenesis by reducing proliferation and increasing apoptosis. These effects result from the suppressed expression of COX-1, COX-2 and iNOS (inducible nitric oxide synthase) [74]. In an arm of the intervention Polyp Prevention Trial, 872 participants were examined to determine the effectiveness of flavonol intake, especially isorhamnetin, kaempferol, and quercetin, which resulted in an inverse association between the serum concentrations of the pro-inflammatory cytokine IL-6. A decrease in IL-6 concentration during the trial was inversely associated with a high risk for advanced adenoma recurrence [75].

Urokinase-type plasminogen activator (uPA) and stromelysin 1 (matrix metalloproteinase 3; MMP-3) are enzymes involved in cancer invasion and metastases including prostate cancer. Quercetin down-regulates uPA and uPAR mRNA expressions [76]. Similarly, the molecule, and other flavonoids, significantly inhibit the *in vitro* invasion of MDA-MB-231 cells in a concentration-dependent manner with an IC_{50} in the micromolar range and reduces MMP-3 activity but not its secretion [77].

Finally, an interesting role for quercetin has been described in the regulation of AMP-activated protein kinase (AMPK), a cellular energy sensor activated by metabolic stresses. Cellular growth and proliferation are processes that demand energy, and AMPK may act as an "energy checkpoint" that allows cells to grow only when energy reserves are sufficient [78]. A number of phytochemicals, including quercetin [41], have been reported to activate AMPK in cell lines and *in vivo* [78,79]. The explanation of how natural compounds possessing different chemical structures can function as AMPK activators probably resides in the observation that many of these molecules activate AMPK indirectly by inhibiting mitochondrial ATP production [78].

6. Conclusions and perspectives

From the data discussed above, quercetin emerges as a molecule possessing multiple properties, which are all directed at ameliorating pathological conditions associated with degenerative diseases, a panacea common to many other naturally occurring compounds. However, in attempting to discriminate between "facts" and "fancies", it is necessary to critically consider some key points in making conclusions regarding quercetin's biological activities and in planning new studies. Uptake, metabolism and circulating concentrations of quercetin and its metabolites suggest that a regular diet cannot provide adequate amounts of quercetin (<1 μM) compatible with any described chemopreventive and/or cardioprotective effect. However, we report here that it is relatively easy to increase total quercetin concentrations in plasma (above 10 μM) by supplementation with quercetin-enriched foods or supplements. Also, in this case, the

Table 3
Selection of studies on quercetin administration in human subjects.

Cohort	Dose	Endpoints	Results	Reference
Healthy volunteers	500–1000 mg/day	Evaluate plasma quercetin concentration after 12 weeks supplementation	6 to 10 fold increase of plasma quercetin concentration compared with placebo	[94]
Healthy volunteers	500 mg/day	Effects of quercetin on CYP2A6, CYP1A2; N-acetyl-transferase; xantine-oxidase activities	Quercetin modulates CYP2A6, CYP1A2; N-acetyl-transferase and xantine-oxidase enzyme activity *in vivo*.	[95]
Healthy volunteers	1000 mg/day with/without EGCG, isoquercetin, eicosopentanoic acids	Quercetin effects on mitochondrial biogenesis and immunity.	Increase in plasma quercetin concentration. Quercetin increase granulocyte oxidative burst activity. Decrease of IL-6; IL-10; CRP	[96]
Healthy volunteers	325 μmol Q-3G 331 μmol Q-4′G	Bioavailability of Q-3G and Q-4′G	Plasmatic bioavailability: 5 μM Q-3G; 4.5 μM Q-4′G	[97]
High cardiovascular disease risk subjects	150 mg/day	Serum lipid levels and blood pressure responses in overweight patients.	Quercetin decreases systolic blood pressure, plasma oxidized LDL and TNF-α in some subjects.	[98]
Overweight and obese subjects with metabolic syndrome traits	150 mg/day	To evaluate the effects of quercetin supplementation on markers related to metabolic syndrome.	Quercetin decreases plasma oxidized LDL and systolic blood pressure.	[26]
Sarcoidosis	4 × 500 mg/day	To evaluate effects of quercetin supplementation in sarcoidosis patients on markers of both oxidative stress and inflammation.	Increased total plasma antioxidant capacity and reduced markers of oxidative stress and inflammation (TNF-α, IL-10, IL-8)	[80]
Upper respiratory tract infection (URT)	500–1000 mg/day	Quercetin supplementation and influences on upper respiratory tract infection	Reduction in URT in middle ages and older subjects	[99]
Rheumatoid arthritis	166 mg plus 133 mg Vit-C	Effects of quercetin on the level of plasma inflammatory biomarkers.	No change in blood biomarkers of inflammation (TNF-α; IL-6; IL-β; CRP)	[100]

efficacy of this molecule cannot be measured in healthy individuals but in subjects affected by diseases. This is the case discussed above for hypertensive patients but also for subjects suffering from sarcoidosis, a chronic inflammatory lung disease [80] (Table 3). These two pathological conditions have a common origin linked to inflammation and oxidative stress, which suggests the need to observe the positive effects of antioxidant supplementation not in healthy subjects but in individuals with enhanced alterations in redox balance and inflammatory markers. This conclusion is also in agreement with the *in vitro* and *ex vivo* selective effects of quercetin observed in cancer cells with respect to differentiated or normal cells.

When considering potential clinical applications for quercetin, it is important to differentiate between pharmacological (hundreds of milligrams in concentrated doses) and nutritional doses of the molecule (a few milligrams diluted in the diet). In the latter case, low concentrations do not saturate metabolic pathways that rely on the supply of cofactors, such as UDP-glucuronic acid [81]. As a consequence, circulating, unconjugated molecules are not found in the blood. Only after the intake of large doses, which saturate conjugation enzymes, can a peak of free aglycone be detected in the plasma, usually for only a short period of time. However, in this case, after a few hours following uptake, metabolism and the release of the molecule and its metabolites from different tissues, a large portion of the quercetin is excreted in conjugated forms, which may not be as active as the aglycone. This fate is common for a large portion of dietary polyphenols [81]. Alternatively, the molecule may be administered by intravenous injection to avoid the formation of conjugates that can reduce the bioavailability of the active moiety and dramatically alter their pharmacological properties. The correct dose will also determine the primary site of metabolism with high doses primarily metabolized in the liver and low doses in the intestines.

In the above paragraphs, we discussed that quercetin targets multiple biochemical signals that control tumor cell proliferation, programmed cell death (including apoptosis), and autophagy, which depends on the cellular model used. At concentrations and times of incubation above 40–50 μM and 24 h, respectively, the effects on cell cycle arrest, autophagy or senescence induction become negligible compared to the appearance of the phenomenon of apoptosis. However, several critical points must be resolved before developing a potential clinical and/or chemopreventive use of the molecule: (1) The mentioned doses are reachable only following pharmacological administration, which necessarily implies potential toxic quercetin effects; (2) In many cases discussed in the present commentary and by others, the biological effects of quercetin are not specific but are common to other flavonoids and/or phytochemicals; (3) A large portion of the studies cited above describe the final effect of quercetin (or its metabolites) on the hallmarks of cancer, but the "first hits" of the molecule *in vivo* remain largely unknown. A contradiction exists between the few "direct" molecular targets of quercetin so far identified (Table 2) and the functional pleiotropy of the molecule, which suggests the existence of many substrates. Despite these obstacles, few cases are emerging where quercetin responds to the following characteristics: (1) efficacy on a specific pathology, (2) low doses applied, and (3) knowledge of its mechanism of action. We refer to chronic lymphocytic leukemia where quercetin, in preclinical models, is applied at concentrations that, *per se*, are not toxic, and apoptotic to neither leukemic B-cells nor normal lymphocytes. However, the molecule is active in bypassing the acquired apoptotic resistance of the malignant cells and increases the efficacy of traditional and innovative chemotherapeutic protocols. From a molecular point of view, we are working on the experimental hypothesis that apoptotic resistance in these cells is induced by the anomalous activity of death receptors, which

results in an enhanced expression, often NF-κB dependent, and activity of anti-apoptotic factors. Quercetin would be able to re-establish sensitivity to DR-dependent pathways by inhibiting the expression of anti-apoptotic factors and/or accelerating their degradation.

Unfortunately, no clinical trials have yet been published on cancer patients using quercetin in monotherapy or in combination with other chemotherapeutic drugs (Table 3). In many cases, quercetin has been administered to healthy volunteers in order to establish its availability and metabolism. Few clinical studies in patients, as mentioned above, refer to the application of the molecule in inflammatory-prone diseases (Table 3). In searching clinical trial databases[4], studies involving quercetin in cancer therapy are still in the recruiting phase, or data are not yet available.

Further research on the possible use of quercetin in adjuvant cancer therapy is extremely warranted. This goal can be achieved only by planning future large-scale clinical trials to ascertain the full chemopreventive and chemotherapeutic efficacy of the molecule.

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