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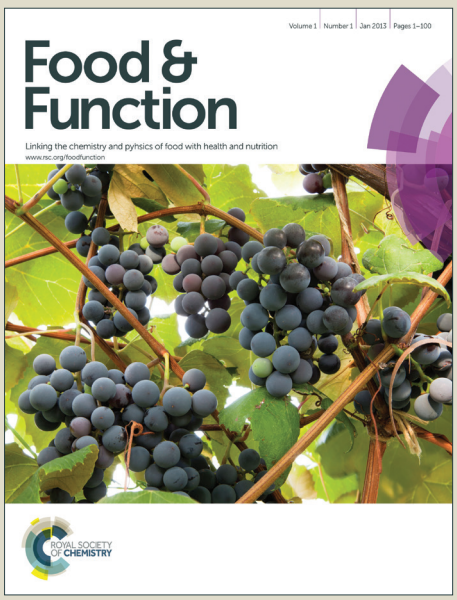

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The pleiotropic flavonoid quercetin: from its metabolism to the inhibition of protein kinases in chronic lymphocytic leukemia

Gian Luigi Russo*, Maria Russo, Carmela Spagnuolo

Istituto Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, 83100, Avellino, Italy

***Corresponding author:**

Gian Luigi Russo

Istituto di Scienze dell'Alimentazione, CNR

via Roma 64, 83100 Avellino, Italy

Phone: +39 0825 299331

Fax: +39 0825 781585

e-mail: glrusso@isa.cnr.it

Abstract

Quercetin is a flavonoid, subclass flavonols, possessing potential anticancer properties. It has been often defined as a functionally pleiotropic molecule, since it can simultaneously target multiple pathways bypassing or ameliorating the onset of drug resistance in malignant cells. In this context, we reviewed the sometime paradoxical antioxidant properties of quercetin and the functional role of its glucuronide and/or sulfate conjugates to discuss the low bioavailability of the molecule measured *in vivo*. We recently demonstrated that the quercetin is able to sensitize several leukemia cell lines as well as B-cells isolated from patients affected by chronic lymphocytic leukemia (CLL) to death ligands agonists (anti-CD95 and rTRAIL). The flavonol also potentiates the effect of canonical and innovative chemotherapeutic drugs (fludarabine and ABT-737) against CLL. The apoptosis-enhancing activity of quercetin in cell lines and B-CLL cells depends upon the modulated expression and activity of Mcl-1, an anti-apoptotic proteins belonging to the Bcl-2 family. Here, we suggest that the pleotropic activity of quercetin in CLL is obtained by direct inhibition of key protein kinases which positively regulate Mcl-1 activity and by indirect down-regulation of Mcl-1 mRNA and protein levels acting on its mRNA stability and proteasome-mediated degradation. Finally, we highlighted the pros and cons of quercetin supplementation in cancer therapy and in prevention.

Key words: polyphenols; flavonoids; quercetin; protein kinases; antioxidant; chemoprevention; chronic lymphocytic leukemia

Abbreviations: v-Akt murine thymoma viral oncogene homolog 1/protein kinase B (Akt/PKB); B-cell lymphoma-2 (Bcl-2); Casein kinase 2 (CK2); Electrophile response element, (EpRE); Extracellular signal-regulated kinase 1/2 (ERK1/2); Glycogen synthase kinase-3 (GSK-3); Keap1, kelch-like ECH-associated protein 1; Mitogen-activated protein kinase kinase 1 (MEK1 or MAP2K1); Myeloid cell leukaemia-1 (Mcl-1); Nuclear factor (erythroid-derived 2)-like 2 (Nrf2); Peripheral blood mononucleated cell (PMBC); Phosphatase and tensin homolog (PTEN); Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI_3K); Quercetin 3-*O*-glucuronide (Q3GlcA); Quercetin–quinone (QQ); Reactive oxygen species (ROS); TNF-related apoptosis-inducing ligand (TRAIL).

Introduction

The term phytochemicals generally indicate a large group of non-nutritional compounds (> 10,000) often associated to the prevention of degenerative pathologies, such as cancer, cardiovascular and neurodegenerative diseases [1-4]. The large part of phytochemicals is represented by polyphenols which, in turn, are classified into two major types: flavonoid and non-flavonoid phenolics (essentially phenolic acids, stilbenes and lignans) [5]. The formers count more than 6,500 different compounds [6] with the common chemical structure consisting of two benzene rings linked through a heterocyclic pyrone C ring (Fig. 1) and divided into the subclasses of: anthocyanins; chalcones; dihydrochalcones; flavanols (also called catechins); flavanones; flavones; flavonols; isoflavonoids [7, 8]. Quercetin (3,3',4',5,7-pentahydroxyflavone) (Fig. 1) is the major dietary flavonol found in fruits, vegetables and beverages, such as tea and red wine [9]. This molecule has been employed in different studies aimed to demonstrate its antioxidant, anti-inflammatory, anti-angiogenic, anti-proliferative and pro-apoptotic effects [10-12]. As we will more extensively discuss in the next paragraphs, the dietary intake of quercetin, as aglycone or in its different glycosylated forms (Fig. 1), is limited to few milligrams per day and the circulating concentrations of the molecule and its metabolites are extremely low (nanomolar range or below) and very far from those required to exert any biological activity. Despite these limits, a recent search on PubMed retrieved more than 2,000 articles in the last 10 years with the term “quercetin” included in the title and the trend over the years is growing. The interest for this molecule probably resides in its pleiotropic nature, an attribute common to other well-known and intensively investigated polyphenols, such as resveratrol, green tea catechins, etc. When applied to biological compounds, the Greek etymology of ‘‘pleiotropy’’, which means ‘‘in more places’’, positively indicates the capacity to trigger simultaneously multiple cellular intermediates of different pathways which converge on common targets. In such a way, it is possible to hypothesize that even at a very low intracellular concentration, the largely discussed anticancer efficacy of quercetin [11, 12] can be the result of an additive or synergistic effects, deriving

from its pleiotropy, which generates a domino effect, leading to arrest or retard cancer growth. This hypothesis can also explain how the combination of several phytochemicals, as normally happens in a diet rich in fruit and vegetables, might be more protective than single compounds, since low doses minimize toxicity without reducing efficacy which is warranted by their multiple modes of action [13, 14].

The pleiotropic functions of quercetin and other phytochemicals depend upon the simultaneous occurrence of several positive events, all going in the same direction: i. uptake of the active form of the molecules; ii. intracellular concentration in a range (usually micromolar) compatible with the affinity constants required to bind their supposed targets; iii. resistance to chemical and biochemical modification within the cell (i.e., phase I and II enzymatic systems); iv. early effects, e.g. capacity to bind cellular target in a very short time (within minutes); v. absence of simultaneous, counteracting effects which may reduce or cancel the primary aim. The latter case is well illustrated by examples regarding quercetin itself: the molecule can be protective against hydrogen peroxide-induced cytotoxicity and apoptosis at lower concentrations, but, on the same cell line, can induce cytotoxicity, DNA fragmentation and caspase activation at higher concentrations [15]. If the two events coexist at intermediate concentrations, the result is a null effect.

In the present review, we will focus on key aspects of the anticancer effect of quercetin that can be explained recurring to its pleiotropic capacity.

Who does the job ? Quercetin or its metabolites ?

Phenol Explorer web site currently represents the most comprehensive and updated database on polyphenol content in foods [9, 16]. Searching for “quercetin” is an useful exercise to evidence the differences existing between the concentrations present in the foods of origin and those circulating in the bloodstream in terms of aglycone, glycosides and metabolites. It is worthwhile to note that

the large majority of data present in the literature refer to "total" concentration of quercetin in food, tissues or blood without discriminate between the free aglycone and its conjugated form. This may generate confusion when the biological activity of quercetin is measured *in vivo* after oral supplementation. In the Western population the estimate daily intake of total flavonols is in the range of 20-50 mg/day, about 13.82 mg/day correspond to quercetin glycosides [17]. The biological consequences of the abundance of quercetin glycosides in food and beverage compared to quercetin aglycone are important for its absorption and metabolism. In fact, the partition coefficient (log octanol/water) is in favor of quercetin aglycone (of 1.2 ± 0.1), compared to its glycosides (0.37 ± 0.06) which show greater hydrophilicity [18]. This means that, since the large part of quercetin in foods is present in glycosylated forms, the hydrophilic moiety must be removed by cellular glycosidases to allow passive diffusion of the molecule across biological membranes.

More than 20 quercetin glycosides have been described; those mostly present in foods as glucosides, such as 3-*O*-Glucoside, 4'-*O*-Glucoside, 3,4'- *O*-di-Glucoside (Fig. 1). Another well-known quercetin glycoside is rutin (quercetin-3-*O*-rutinoside; Fig. 1). Hydrolysis of quercetin glucosides to aglycone occurs in saliva, although epithelial cells in the small intestine represent the main site for the hydrolysis of quercetin glucosides by the activity of lactase phlorizin hydrolase on the cell surface and/or intracellular β-glucosidase [19-21].

After absorption, quercetin is firstly metabolized in the cells of the small intestine. Here, the molecule is conjugated to methyl and sulfate groups and glucuronic acid to generate its major conjugates in humans: 3-*O*-methyl-quercetin (isorhamnetin), quercetin-3-*O*-glucuronide (Q3GlcA), isorhamnetin 3-*O*-glucuronide, quercetin-3'-*O*-sulfate and quercetin glutathione conjugate [22-24] (Fig. 1). Other organs where quercetin aglycone conversion may occur are large intestine, colon, liver and kidney. Across the hepatic portal vein, quercetin metabolites reach the liver where metabolic conversion is completed. Certainly the liver possesses all the enzymatic systems necessary to metabolize quercetin, as demonstrated using several excellent cell free models [25, 26]; however, if the large part of the aglycone is converted at the intestinal level, what is the effect of phase II enzymes

on quercetin metabolism into the liver and the other organs? Major interorgan and interspecies differences in phase II metabolism of quercetin exist since in humans, in contrast with rats, methylation takes place mainly in the liver, whereas sulfation of quercetin is mainly performed by the intestine [26]. In addition, in hepatic cell free systems, sulfated and glucuronidated metabolites of 4'-*O*-methyl-quercetin and 3'-*O*-methyl-quercetin have been identified [26]; in HepG2 cells, quercetin-7- and quercetin-3-glucoronides can be methylated or hydrolyzed by endogenous β-glucuronidases followed by sulfation to quercetin-3'-sulfate [27]. We should also consider the possibility that a portion of the quercetin aglycone and/or quercetin glycosides escapes intestinal transformation and is metabolized at hepatic level. Is this hypothesis rationale ? Certainly, in contrast to dietary intake, pharmacological doses of quercetin can saturate the intestinal conjugating phase-II enzymes and pass into the hepatic portal vein in a free unconjugated form [27]. Alternatively, the absence of quercetin glucosides in the circulation [28, 29] and the observation that human liver possesses β-glucosidase activity [30] suggest that liver phase II enzymes can transform quercetin glycosides which escaped intestinal metabolism. From this view emerged a still unclear and partially unknown interplay of quercetin metabolites between intestine, liver and other organs in terms of uptake, efflux and bioconversion. This remains a key point since the regioselectivity of phase II conjugations is of great importance to determine the biological activity of quercetin which is known to be dependent on the number and position of especially unconjugated free hydroxyl groups [31].

Two corollarial deductions of the above discussed issues are: i. none of the *in vitro* models is able to fully convert quercetin to a phase II metabolite mixture similar to plasma metabolite pattern of quercetin present *in vivo* [26, 27]; ii. no quercetin aglycone or its glycosides can be present in the bloodstream following nutritional doses. The latter conclusion have been reached after intense debates [28]. To summarize the results of the interventional study performed on human subjects supplemented with quercetin, its glycosides or quercetin-enriched food as reported in several excellent reviews, the maximal concentration in plasma ranged between 0.14-7 μM resulting from

an ingestion of quercetin or quercetin equivalents of 0.008 - 4 gr [28, 32, 33]. Quercetin is not found in the plasma as aglycone or as the parent glucosides since, at the doses generally employed in the intervention studies, it would be found exclusively as methyl, sulfate, or glucuronic acid conjugates. These conclusions make a paradox which can be extended to other biologically active phytochemicals: if the free quercetin aglycone is absent *in vivo* after a dietary intake or supplementation with high doses, how can we explain the large biological activity of the molecule largely described *in vitro*? Two main hypotheses can be considered which may coincide, both supported by experimental data: i. conjugated forms of quercetin are biologically active; ii. after cellular uptake, quercetin metabolites are de-conjugated regenerating the free aglycone. De-conjugation of quercetin can occur in liver [27], in fibroblasts [34], in human neutrophils during inflammation [35] leading to reactivation of the molecule. The number of papers describing an active role of quercetin metabolites *per se* are rapidly growing. Plasma antioxidant status is significantly higher in animals to which quercetin is administrated, suggesting that quercetin metabolites can retain some antioxidant activity when the o-catechol group does not undergo conjugation reactions [36]. In many cases, the beneficial activities of quercetin metabolites result in ameliorating the response to oxidative stress following inflammation. As an example, Q3GlcA appears more effective than its aglycone in the protection against oxidative stress induced by the extracellular attack of ROS, because its higher chemical stability [20]. In fact, Q3GlcA at physiological level (1 μM) efficiently inhibits peroxynitrite-induced nitrotyrosine formation in human serum albumin [37], exerts antioxidant activity more effectively than quercetin aglycone in 3T3 mouse fibroblast exposed to hydrogen peroxide [38] and is a potential inhibitor of myeloperoxidase at physiological concentrations against neutrophil-mediated low-density lipoprotein oxidation [39]. It has been postulated that inflammation enhances intracellular β-glucuronidase leading cells exposed to glucuronide conjugates of quercetin to release their aglycone [20]. In contrast, a recent article suggests that Q3GlcA did not affect inflammatory gene expression, since proinflammatory microRNA-155 is down-regulated by quercetin and isorhamnetin but not by Q3GlcA [40]. In vascular systems, quercetin

and its conjugated metabolites, at physiologically achievable concentrations, modulate vascular function in human and animal models comprising vascular endothelial and smooth cells [41-43]. To our knowledge, only one report has been published on the antiproliferative effects of quercetin metabolites (Q3GlcA and quercetin-3'-sulfate) in malignant cells, significantly increasing PPAR-γ expression [44]. A recent work on cell lines of different origin suggested that conjugation of quercetin with glucose or glucuronic acid eliminated the anti-proliferative effects of the aglycone, while methylated and sulphated metabolites (isorhamnetin and quercetin-4'-O-sulphate, respectively) decreased its anti-proliferative effects, but maintained some anti-proliferative activity [45].

In this scenario, key issues remain to be addressed: i. to determine the efficacy of the mechanisms of uptake of quercetin metabolites must be clarified; ii. to assay the substrate specificity of each metabolite which is largely unknown; iii. to establish if all biological activities of quercetin metabolites require a preliminary de-conjugation to the aglycone form. The use of pure compounds tested in *in vitro* models is welcomed to shed light on these questions.

Quercetin is a pleiotropic kinase inhibitor

Exhaustive reviews on quercetin and its role in cancer and other degenerative diseases frequently appear in the current literature [10, 12, 24, 46-48]. These recent articles well describe the different properties of quercetin which cover many ‘‘hallmarks of cancer’’, including the antiproliferative and growth-suppressing effects, the induction of senescence and telomerase inhibition; the induction of cell death and autophagy, the anti-angiogenic activity, the activation of immune destruction reviewed elsewhere [12]. To our knowledge, no clinical trials on the administration of quercetin to cancer patients in monotherapy or in combination with other chemotherapeutic drugs have been published so far; therefore, all the conclusions on the potential chemopreventive and

chemotherapeutic use of quercetin *in vivo*, must take in account the criticisms discussed above on its metabolism, tissue distribution and uptake.

Recently, we and other reviewed the ability of quercetin to inhibit protein kinases regulating cell growth in cancer cells [32, 49]. Early works demonstrated that quercetin was able to inhibit both tyrosine and serine-threonine kinases [50-52]. However, the direct binding between quercetin and the inhibited kinases has only been reported in a few cases and linked to a cause-effect mechanism responsible for growth suppression [32]. The cellular kinases directly targeted by quercetin are: MEK-1, $PI_3K\gamma$, IKK α/β, Hck (Src tyr kinase family), GSK-3, CK2 (Table 1 and reference [32]). It is worthwhile to note that *in vitro* screenings identified more than 100 kinases inhibited by greater than 95% at 30 μM by quercetin, very close to the mean IC_{50} calculated for the molecule in many cell lines [53]. At lower concentrations (2 μM), quercetin decreased the activity of ~15 kinases by greater than 95% and that of a remaining set of ~50 kinases by 80-95%, suggesting that these kinases may represent specific targets for quercetin. With a similar approach, 34 kinases obtained after tissue purification or expressed in prokaryotic or eukaryotic systems, have been earlier identified as potential substrates for quercetin inhibition [54] with a decrease of the enzymatic activities by less than 30% at 20 μM concentration. However, discrepancies emerged between these *in vitro* large screenings and studies on single kinases, suggesting that the inhibitory effect measured must be confirmed on native kinases in cellular models and associated with deregulation of cell growth or other biological effects [32].

In the next paragraphs, we will focus our attention on MEK-1 and $PI_3K\gamma$, since their inhibition by quercetin can magnify the concept of quercetin as a pleiotropic multi-kinase inhibitor (Table 1). Quercetin interferes with the PI_3K-Akt/PKB and RAS/RAF/MEK/ERK pathways which inhibit apoptosis, increase proliferation and cellular growth, invasion, and metastasis. In the first case, X-ray crystallographic structure of $PI_3K\gamma$ bound to quercetin indicates that the molecule fits into the ATP binding site with a Kd value of 0.28 μM [49, 55]. The direct binding of PI_3K by quercetin inhibits downstream the PI_3K-Akt pathway of signaling including inhibition of AP-1 and NF-κB activation

[56, 57]. In the case of RAS/RAF/MEK/ERK pathway, structural studies indicate that quercetin can directly bind with Raf-1 and MEK-1 *ex vivo* and *in vitro*, with stronger inhibition of MEK-1 kinase activity than Raf-1 [58]. In this case, quercetin seems to bind in a pocket separate from, but adjacent to the ATP-binding site of MEK-1 [49]. This interaction leads to inhibition of phorbol ester-induced phosphorylation of ERK and $p90^{RSK}$, and the activation of AP-1 and NF-κB. It is interesting that quercetin exerted stronger inhibitory effects than PD098059, a well-known pharmacologic inhibitor of MEK-1 [49, 58].

We will discuss now how the inhibition of these pathways can act in concert to ameliorate resistance to chemotherapeutic drugs in chronic lymphocytic leukemia (CLL).

Pleiotropic effect of quercetin in CLL

Our interest for quercetin in leukemia is dated more than a decade ago, when we demonstrated that the flavonol, in association with the agonistic antibody anti-CD95, able to bind and activate the death receptor CD95/Fas/Apo-1, induced apoptosis in HBP-ALL cells, a lymphoblastic leukemia cell line highly resistant to apoptogenic induction [59]. The involvement of quercetin in leukemia was not new, since the group of Larocca and co-workers already described that quercetin inhibited the clonogenic activity of several acute myeloid and lymphoid leukemias (AML and ALL) inducing the expression of transforming growth factor-β 1 in leukemic blasts as one of the growth-inhibitory mechanism [60]. Our novel contribution was that no toxic doses of quercetin significantly ameliorated the apoptotic resistance when anti-CD95 was added. In other words, quercetin mono-treatment was neither cytotoxic, nor apoptotic, but in association with pro-apoptotic agents enhanced their efficacy in inducing apoptosis in leukemic cells. This finding has been confirmed in several human leukemia cell lines of lymphoid or myeloid origin and extended to another death ligand, namely rTRAIL (recombinant TRAIL) able to bind the TRAIL (TNF-related apoptosis-inducing ligand) receptor [61].

These observations represented the initial rationale to explore the effects of quercetin in CLL. In fact, CLL, the most frequent form of leukemia in adults in the Western world with an incidence which oscillates annually between 3.5 and 6.15 cases per 100,000 habitants, is characterized by a patient survival for years or decades without any treatment because of the relatively slow progression rate of the disease. Unfortunately, other patients can experience a rapid and fatal disease despite therapy, depending on the clinical staging (Rai or Binet) which classify patients according to tumor burden and hematopoietic impairment [62-64].

CLL is considered a highly heterogeneous and still an incurable disease; patients with active symptoms or with advanced Binet or Rai stages require therapy, but most treated patients become resistant to the first-line chemotherapeutic treatment (e.g. fludarabine, cyclophosphamide and rituximab) and show apoptotic resistance. We firstly demonstrated that in B-cells isolated from peripheral blood of CLL patients and resistant to apoptosis induced by anti-CD95 and rTRAIL, the addition of quercetin, in combination with anti-CD95 and rTRAIL, significantly enhanced apoptosis when compared to mono-treatments with quercetin or death ligand agonists single treatment [65]. Subsequently, we demonstrated that quercetin could also strengthen the efficacy of fludarabine. In fact, B-CLL resistant to 3.5–14 μM fludarabine (a range of values corresponding to the therapeutic plasma concentration of fludarabine) showed an increase in cell death by approximately two-fold in the combined treatment when compared with quercetin and by six-fold respect to fludarabine [65]. Overall, these data demonstrated that the presence of quercetin, only when associated with first-line therapeutic drugs (e.g., fludarabine) or death receptor inducers, ameliorated sensitivity to apoptosis in B-CLL normally resistant to cell death. More recently, we reported that the association between ABT-737 and quercetin synergistically induces apoptosis in B-cells and in five leukemic cell lines [66]. ABT-737 is a so-called BH3 mimetic agent, i.e., a small molecule which binds with high affinity (<1 nM) to Bcl-2, Bcl-X_L and Bcl-w, members of the family of the Bcl-2 (B-cell lymphoma-2) anti-apoptotic factors, whose over-expression is considered a hallmark of CLL [67]. In B-cells resistant to ABT-737, quercetin synergistically induced cell death when associated with ABT-737 [66].

Resistance of B-cells to ABT-737 is due to the demonstration that this drug antagonizes several members of Bcl-2 protein family, but shows low affinity for Mcl-1 (Myeloid cell leukaemia-1, discovered as a pro-survival member of the Bcl-2 family), whose increased expression in CLL determine resistance to apoptosis [68]. We hypothesized that, in multiple ways, quercetin could contribute to remove the block caused by Mcl-1 over-expression, allowing ABT-737 to show its therapeutic efficacy. How does quercetin make this effect? In a recent work from our laboratory, we reported that, in U-937 cells (derived from a histiocytic lymphoma), quercetin down-regulates Mcl-1 acting directly or indirectly on its mRNA stability and protein degradation, suggesting that the same mechanism may bypass resistance to apoptosis in leukaemic cells isolated from CLL patients and sensitize B cells to apoptosis induced by drugs and death receptor inducers [69] (Fig. 2). In addition, in both cell lines of leukemic origin and in B-CLL cells, we demonstrated that quercetin down-regulated Mcl-1 protein interfering with PI_3K/Akt and MEK/MAPK pathways [66]. As discussed above and schematically summarized in Fig. 2, quercetin can directly bind to and inhibit PI_3K [55] and MEK [58] which are both positive regulators of Mcl-1 anti-apoptotic activity [70, 71]. Perhaps, the pleiotropic and synergistic effects of quercetin in inhibiting Mcl-1 can be affected by two additional kinases: CK2 and GSK-3, both are direct target for quercetin inhibition with an IC_{50} of 0.92 and 2 μM, respectively [32, 49, 72, 73] (Table 1) and both can regulate Mcl-1 activity, although in opposite manners. CK2 is a potential activator of PI_3K/Akt pathway since it phosphorylates and inactivates PTEN (phosphatase and tensin homolog), which, in turn, inactivates PI_3K [74]; therefore, inhibition of CK2 activity by quercetin may contribute to switch off PI_3K/Akt pathway (Fig. 2). On the opposite, GSK-3 is a negative regulator of Mcl-1 since it phosphorylates Mcl-1 at S159 triggering its degradation [75, 76]. In this case, a putative inhibition of GSK-3 kinase activity by quercetin could result in an unwanted activation of PI_3K/Akt pathway (Fig. 2). However, it is also known that phosphorylated and active Akt inactivates GSK-3 by phosphorylation on its N-terminal serine. Inactive (phosphorylated) GSK-3 cannot phosphorylate Mcl-1 and drive its degradation, thus stabilized Mcl-1 can promote antagonism of pro-apoptotic factors and prevent cell death [77]. If this is

the case, the potential binding of quercetin to GSK-3 generates a futile cycle. This apparent contradiction is a good example on the caution required before attributing pleiotropic functions to broad kinase inhibitors, whose targets regulate antagonistic processes, such as cell survival and cell death. For CK2 and GSK-3, it must be determined their biochemical and functional *weight* in terms of Mcl-1 activation in CLL and, more importantly, it is mandatory to verify if the two kinases are *in vivo* inhibited by quercetin in B-cells.

In summary, we accumulated evidence that the pleiotropic effects of quercetin in CLL can be the results of the following actions occurring at the same time and in the same cells: i. inactivation of PI_3K-Akt/PKB pathway via direct inhibition of PI_3K kinase; ii. inactivation of RAS/RAF/MEK/ERK via direct inhibition of MEK; iii. destabilization of Mcl-1 mRNA in U-937 cells.; iv. induction of proteasome-dependent degradation of Mcl-1. The net result is the loss of function of anti-apoptotic Mcl-1 in CLL and the consequent sensitivity of B-cells to drugs which induce apoptosis.

The possibility to prolong the period of latency of the disease in patients diagnosed with CLL and classified as Rai stage I/II (asymptomatic) with a chemopreventive intervention based on quercetin supplementation represents the rationale to design future clinical trials. In fact, the limited or absent cytotoxicity of quercetin mono-treatment on both leukemic cell lines and PMBC (peripheral blood mononucleated cell) used as "normal" counterpart of leukemic cells [61, 66] and the well-established tolerability of quercetin when administered orally to healthy subjects as supplement (reviewed in [28]), suggests a potential chemopreventive use of the molecule.

Conclusions

We introduced the present review suggestion a correct use of the term pleiotropy when referred to the capacity of biologically active phytochemicals to hit multiple cellular targets leading to the

desired effect on cellular functions. This can be the case for quercetin in CLL. In fact, the molecule can switch the equilibrium between pro-apoptotic and anti-apoptotic factors towards the former in B-cells. A key issue in this scenario is to study the uptake of quercetin and/or its metabolites in B-cells. Unfortunately, to our knowledge, no published data currently exist on this topic. We very recently reported that in HPB-ALL cells, derived from a T cell leukemia, quercetin applied at the dose of 50 μM was clearly detectable after 1 hour at an approximate concentration of 200 nM. However, its intracellular concentration rapidly decreased after 3 h and became undetectable at 6 h [78]. In addition, preliminary evidence from our laboratory suggest that quercetin is present as aglycone in B-CLL and inhibits PI_3K-Akt/PKB pathway within few hours from treatment at concentrations in the micromolar range, enough to satisfy the K_i of its target substrates (paper in preparation).

A part from the associations between quercetin and death ligands, or quercetin and fludarabine/ABT-737 described above, several examples are reported in the literature on the ability of the molecule to enhance the anticancer properties of chemotherapeutic drugs. This is the case of the co-treatment with cisplatin in ovarian cancer [79, 80], in nasopharyngeal carcinoma [81], in hepatocellular carcinoma [82] and in mesothelioma cells [83]; with carboxyamidotriazole in breast carcinoma cells [84]; with tiazofurin in ovarian carcinoma [85]; with cytosine arabinoside in leukemic cells [86]. These examples are referred to different types of cancer opening the possibility that quercetin may keep a broad capacity to lower the threshold of resistance to cytotoxic and apoptotic drugs in malignant cells other than leukemias acting on regioselective pathways and taking advantage of its pleiotropic functions.

These observations make quercetin a potential agent in combination with first-line treatments and/or adjuvant chemotherapy in CLL and other malignancies. The main obstacle is how to ensure circulating doses of aglycone. Perhaps, pharmacological doses (2-4 g/day) administered orally [11], may saturate the metabolic pathways of conjugation (methylation, sulfatation and glucuronidation) which require the supply of cofactors such as UDP-glucuronic acid and are potentially bioavailable

and biologically active [4, 33]. Alternatively, quercetin must be administered intravenously in patients subjected to chemotherapeutic treatment to avoid that the formation of conjugates in favor of the free aglycone. To this respect, in humans, the unique phase I clinical trial of quercetin so far completed recommended a dose of 1,400 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 [87]. However, if the use of pharmacological doses of quercetin with potential risks of toxicity can be tolerated in cancer patients, it becomes unacceptable in chemopreventive applications and in nutraceutical supplementation. In the latter case, we should count on the possibility that the mixture of quercetin metabolites obtained after intestinal absorption and metabolism are active and/or that, once in the cells, these compounds are de-conjugated to free aglycone. If future studies will confirm this possibility, it will be probably more clear the range of quercetin concentrations required to improve well-being and prevent degenerative diseases [88], versus those necessary for therapeutic use. Before this happens, caution is necessary in considering the enormous number of promising drug delivery systems, such as inclusion complexes, liposomes, nanoparticles or micelles aimed improve quercetin bioavailability [89, 90]. Again, while improving quercetin aglycone bioavailability may represent a desirable goal in therapy, it can be useless or even dangerous in healthy subjects. In fact, the formation of quercetin–quinone (QQ) species have been described when the molecule is employed as an antioxidant [91]. QQs, like other semiquinone radicals and quinones, are toxic because of their ability to arylate protein thiols. Protection against QQ may arise from GSH which, when present at the right concentration, quickly traps QQ [19].

Therefore, scientists must be aware on the possibility that if quercetin (and other phytochemicals) exerts its biological activities in the *wrong* cell line or in an *inappropriate* space-to-time window of the cell physiology, the consequences observed can be opposite to those expected.

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Figure Legends

Fig. 1. Structure of quercetin aglycone and its most representative glycosides and metabolites in humans. Dietary quercetin glycosides are absorbed in the digestive tract, metabolized in the intestinal epithelial cells and in the liver cells by phase II enzymes and transferred to the blood circulation where almost exclusively quercetin conjugates are present (see text for details). Structures of quercetin glucosides and metabolites have been retrieved from Phenol-Explorer web site.

Fig. 2. The scheme summarizes the capacity of quercetin (Q) to synergize with several apoptotic inducers, namely anti-CD95, rTRAIL (structures not reported in figure), fludarabine (F) and ABT-737 (ABT) and enhance apoptosis in B-CLL cells. Data on cell lines and B-cells isolated from CLL patients suggest that key kinases, such as PI_3K and MEK-1, can be inhibited by quercetin leading to inactivation of the anti-apoptotic factor Mcl-1. For CK2 and GSK-3 the potential role of quercetin inhibition has not been clarified yet (see text for further descriptions). Structures of Q, F and ABT have been retrieved from PubChem Compound[1].

[1] http://www.ncbi.nlm.nih.gov/pccompound/

Table 1
Selected kinases involved in Mcl-1 regulation targeted by quercetin

Targets	Binding site	Concentration	Reference
MEK-1	Activation loop	1 - 2 μM	49, 58
$PI_3K\gamma$	ATP-binding site	3.8 μM	55
CK2	ATP-binding site Competitive inhibitor	IC_{50} = 0.92 μM K_i = 1.18 μM	72
GSK-3β	ATP-binding site	IC_{50} = 2 μM	73

quercetin-3-*O*-rutinoside

intake

quercetin-3-*O*-glucoside

quercitrin

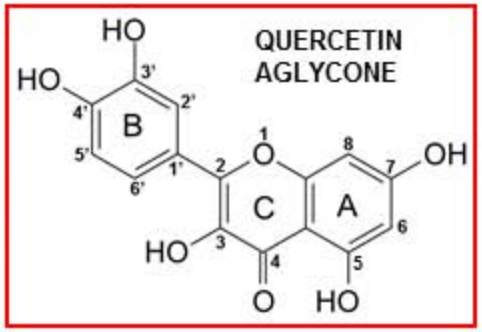
Intestinal tract

quercetin-4'-*O*-glucoside

quercetin 3,4'-di-*O*-glucoside

adsorption

QUERCETIN AGLYCONE



β-Glycosidase
UGT
PST
COMT

portal vein

UGT
PST
COMT
β-Glycosidase

metabolism

blood

8-glutathionylquercetin

quercetin-3'-*O*-sulfate

quercetin-3-*O*-glucuronide

quercetin-3'-*O*-methylquercetin
Isorhamnetin

Fig. 1

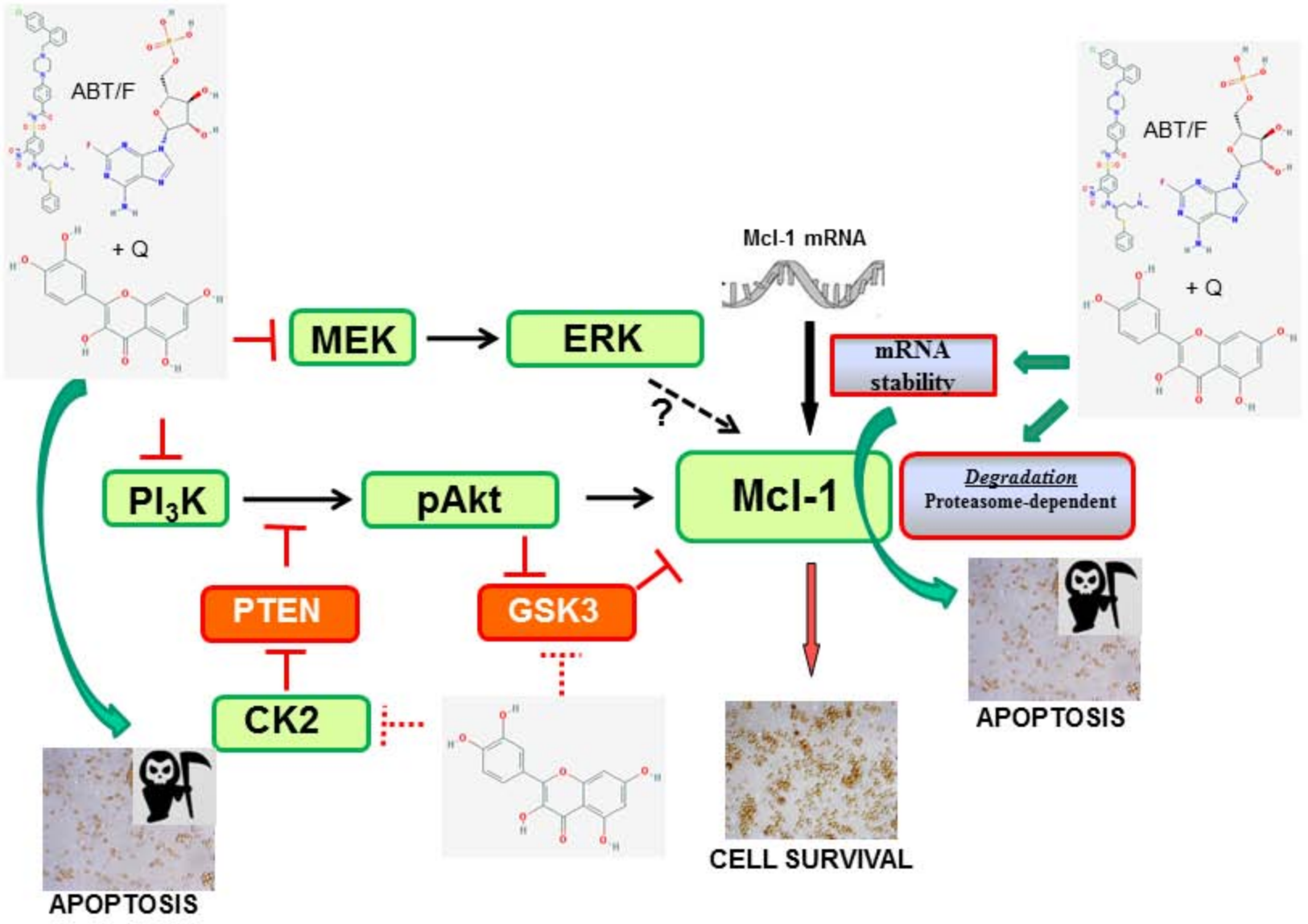


Fig. 2