Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides

Fabio Galvano[(a)], Luca La Fauci[(a)], Paola Vitaglione[(b)], Vincenzo Fogliano[(b)], Luca Vanella[(a)] and Catherine Felgines[(c)]

[(a)] *Dipartimento di Chimica Biologica, Chimica Medica e Biologia Molecolare, Università degli Studi, Catania, Italy*

[(b)] *Dipartimento di Scienze degli Alimenti, Università degli Studi "Federico II", Naples, Italy*

[(c)] *Laboratoire de Pharmacognosie et Phytothérapie, Faculté de Pharmacie, Clermont-Ferrand, France*

Summary. Cyanidin and its glycosides (Cy and Cyg) have been indicated as promising candidates as dietary compounds with a potential role in human health. They are the largest class of water-soluble compounds in plants, where they are responsible for the brillant color (red, orange, blue) of fruits and flowers. As natural compounds of several foods such as vegetables, fruits and red wines, they are estimated to be widely ingested by humans. This paper, basing on the data previously reviewed in 2002, focuses on the findings regarding human and animal studies on Cy and Cyg absorption and metabolism, antioxidant activity and biological properties, with particular attention to anti-carcinogenic activity, vasoprotective, anti-inflammatory, anti-obesity and anti-diabetes effects. It is concluded that although Cy and Cyg bioavailability is low, further investigations are necessary because some important metabolites may still not have been identified. Literature data on antioxidant activity and biological properties, however, widely confirm Cy and Cyg as dietary compounds with a potential beneficial role in human health.

Key words: cyanidin, cyanidin glycosides, biological properties, antioxidant activity.

Riassunto *(Biodisponibilità e proprietà biologiche dell'antiossidante naturale cianidina e dei suoi derivati glicosidici)*. Il ruolo potenzialmente benefico per la salute umana espresso dalla cianidina e dai suoi derivati glicosidici (Cy e Cyg) è confermato da un crescente numero di dati in letteratura. Queste molecole rappresentano la più larga classe dei composti idrosolubili nelle piante, nelle quali sono responsabili del colore brillante (rosso, arancione, blu) dei frutti e dei fiori. Essendo contenuti in diversi alimenti come vegetali, frutti e vini rossi, si stima che siano ampiamente assunti con la dieta umana. Il presente articolo, partendo dai dati precedentemente raccolti nel 2002, focalizza la propria attenzione sulle scoperte riguardanti studi su uomini ed animali inerenti l'assorbimento e il metabolismo di Cy e Cyg, la loro attività antiossidante e le proprietà biologiche, con particolare attenzione alla attività anticarcinogenica, vasoprotettiva, antinfiammatoria, anti obesità ed anti diabetica. Dai risultati analizzati si evince che non è ancora possibile trarre conclusioni definitive sulla biodisponibilità delle cianidine in quanto alcuni importanti metaboliti potrebbero non essere ancora stati identificati. I dati relativi all'attività antiossidante e alle proprietà biologiche, tuttavia, confermano ampiamente il ruolo della cianidina e dai suoi derivati glicosidici come composti dietetici con un ruolo potenzialmente benefico per la salute umana.

Parole chiave: cianidina, glicosidi della cianidina, proprietà biologiche, attività antiossidante.

INTRODUCTION

The anthocyanins (Greek antos, flower and kyanos, blue) are part of the very large and widespread group of plant constituents known collectively as flavonoids. They are water-soluble glycosides of polyhydroxy and polymethoxy derivates of 2-phenylbenzopyrylium or flavylium salts. In fruits and vegetables there are six basic anthocyanin compounds. The differences between individual anthocyanins are the number of hydroxyl groups in the molecule; the degree of methylation of these hydroxyl groups, the nature, number, and location of sugars attached to the molecule; and the number and the nature of aliphatic or aromatic acids attached to the sugars in the molecule [1-3]. All these variables account for the large number of compounds belonging to the

Indirizzo per la corrispondenza (Address for correspondence): Fabio Galvano, Dipartimento di Chimica Biologica, Chimica Medica e Biologia Molecolare, Università degli Studi, Viale Andrea Doria 6, 95125 Catania, Italy. E-mail: fgalvano@unict.it.

Table 1 | *Human studies of cyanidins bioavailability*

Source	Cy and Cyg	Ingested amount (mg)	Plasma C_{max} (nmol/L)	Plasma T_{max} (h)	Urinary excretion (% of intake)	Urine T_{max} (h)	Duration of urinary collection (h)	References
Elderberry extract	Cy 3-samb, Cy 3-glc	720			0.077		4	[15]
Blackcurrant juice	Cy 3-rut,	716-1239		~ 0.75	0.048-0.072		4	[7]
Blackcurrant concentrate	Cy 3-rut, Cy 3-glc	188			0.064	≈ 2	7	[6]
Boysenberry concentrate	Cy 3-glc, Cy 3-soph	344			0.029	≈ 2	7	[6]
Elderberry juice concentrate	Cy 3-samb, Cy 3-glc	3570			0.053	≈ 1	5	[8]
Elberberry extract	Cy 3-samb, Cy 3-glc	147	~ 27	1.5	0.37	1.5	7	[25]
Blackcurrant juice	Cy 3-rut	145	~ 5.7	1	0.04	1.5	7	[25]
Purple sweet potato beverage	Acylated Cy	311	~ 2.3	~1.5	0.01-0.03 [a]		24	[9]
Purple carrots	Acylated Cy glycosides	416	5.8	~ 2	0.030	≈ 4	24	[12]
Blackberries	Cy 3-glc	418			0.16	2-4	24	[18]
Hibiscus sabdariffa extract	Cy 3-samb	147	~ 5.6	1.5	0.018	1.5	7	[5]
Freeze-dried black raspberries	Cy 3-rut, Cy 3-xylrut	1440	~ 56	1-2.5	0.032-0.045 [a]	0-4	12	[14]
Chokeberry extract	Cy 3-gal, Cy -3-ara	721	96	2-4	0.15	3-4	24	[19]
Elderberry juice	Cy 3-samb, Cy 3-glc	361-722	~ 95	~ 1	0.033-0.040 [a]	0.5-1.5	7	[13]
Concentrated elderberry juice	Cy 3-samb, Cy 3-glc	3570			0.06	0.50	24	[11]

Cy: cyanidin. a: depending on the cyanidin considered in the mixture.

anthocyanin family and allow the researchers to study fingerprints of many different vegetables species, just on the basis of their anthocyanin composition. They are of great nutritional interest because of the marked daily intake (180 to 215 mg/day in the United States) [2], which is much higher than the intake (23 mg/day) estimated for other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin [2]. They have been reported to have positive effects in the treatment of various diseases [3] and are prescribed as medicines in any countries. The anthocyanin-health properties are due to their peculiar chemical structure, as they are very reactive towards reactive oxygen species (ROS) because of their electron deficiency. In the last years, great attention was given to the possible protection exerted by natural antioxidants present in dietary plants, particularly flavonoids and polyphenols, towards tissue injury mediated by ROS. Anthocyanins are included in the list of natural compounds known to work as powerful antioxidants. Since cyanidin and its glycosides (collectively: Cy and Cyg) represent one of the major groups of naturally occurring anthocyanins their antioxidant and biological properties have been deeply investigated [4] and recent findings, indicating the possibility that anthocyanins are absorbed as glycosides, have renewed the interest for the studies on their bioavailability, including their absorption, metabolic fate and excretion. Cy are considered the widest spread anthocyanin in the plant kingdom. They are largely distributed in the human diet through crops, beans, fruits, vegetables and red wines, suggesting that we daily ingest significant amounts of these compounds from plant-based diets. Various authors, using different analytical methods, have reported the presence of Cy in many fruits and vegetables. However, almost the totality of the studies does not report quantitative estimation of Cy and Cyg [4].

BIOAVAILABILITY

Human studies

Basing on the data review by Galvano *et al.* [4], the results of a literature survey since 2002 on Cy and Cyg bioavailability in humans are presented in *Table 1*. In the studies here reported, single doses of 188-3570 mg total Cyg were given to the volunteers, most often in the form of berries, berry extracts, or juices. After such intakes, maximal plasma anthocyanin concentrations were very low, on the order of 2.3-96 nmol/L. The mean time to reach these concentrations was around 1.5 hour. Most studies reported very low relative anthocyanin urinary excretions, ranging from 0.018 to 0.37%. Maximal urinary Cy excretion is usually achieved in less than 4 h. The most striking features of these surveys are thus that Cyg are very quickly absorbed and urinary excreted and that the fraction of orally administered Cy excreted in urine is very low.

Many studies have only detected unchanged anthocyanin glycosides in both plasma and urine [5-14]. However, these last years, methylated derivatives, anthocyanidin glucuronide conjugates as well as an-

thocyanin glycoside glucuronides have been identified in urine or plasma by the use of HPLC combined with mass spectrometry [15-19]. Kay *et al.* [17] have also identified an oxidized derivative of anthocyanins.

Methylation at the 3'-OH moiety of various CY glycosides (glucoside, galactoside, xyloside, sambubioside) has been largely reported [15, 17-19, 20]. Recently, Tian *et al.* [20] observed that methylation potentially occurred at both the 3'- or 4'-hydroxyl position of a triglycoside anthocyanin (Cy 3-xylosylrutinoside) whereas mono- or diglycosides gave rise to only one methylated derivative.

Formation of monoglucuronides of Cy and its methylated derivative peonidin has been observed after various berry anthocyanin feeding [15, 16, 18, 19]. After blackberry consumption, two monoglucuronoconjugates of Cy have been identified [18]. Moreover, a Cy diglucuronide has been identified in urine [18]. Wu *et al.* [15] have also identified a Cy 3-glucoside glucuronide in urine from elderly women after consumption of an elderberry concentrate. The exact sites of Cy glucuronidation are still unknown. Two possible pathways could explain the formation of monoglucuronides of Cy and Cyg. A possible pathway is that Cyg were hydrolyzed to aglycones then rapidly glucuronidated by an UDP-glucuronosyltransferase in the intestine. On the other hand, another possible pathway is that Cyg could serve as a substrate for UDP-glucose dehydrogenase to form Cy glucuronides. Indeed, such an enzyme is present in both the small intestine and liver [21]. This last hypothesis does not require hydrolysis to aglycones, which are unstable at physiological pH. Therefore, it could be regarded as a principal glucuronidation pathway and could thus result in the formation of major metabolites.

Small amounts of sulfoconjugates of Cy have been identified in urine [18]. Cy sulfoconjugate formation requires hydrolysis of glucoside to the aglycone and then sulfoconjugation of the aglycone by sulfotransferases present in numerous tissues, including the intestine and liver [22]. Furthermore, aglycone (Cy) has been detected in urine samples analyzed immediately after their collection [18]. Given that aglycone is very unstable at physiological pH, it is unlikely that it all arise from the small intestine. Small amounts of aglycone could thus be released from conjugates by β-glucuronidases and sulfatases present in both kidney and urine [23, 24].

Proportion of metabolites (methylated and/or glucuronides) related to total anthocyanins excreted in urine was estimated to around 6% [25], 25% [15], 68% [19] or higher than 85% [18]. Some of these discrepancies could arise from sample conservation, extraction procedures as well as the sensitivity and resolution of HPLC methods.

The glycoside moiety may have an influence on the absorption of Cyg. Indeed, Nielsen *et al.* [7] have found a higher plasma concentration and urinary excretion of Cy rutinosides than of Cy glucosides in relation to dose.

Animal studies

Cyg bioavailability, evaluated by urinary excretion, is low: in rats, 24-h urinary excretion was estimated around 0.19-0.26% of the ingested dose after feeding blackberry or bilberry Cyg [26-28]. This excretion is of the same order of magnitude after rats have ingested acylated anthocyanins [9, 29]. Anthocyanin urinary excretion was found to be ~0.36% 48 h after blackcurrant juice administration to rabbits [7]. In weanling pigs, urinary total recovery of Cy derivatives was 0.087% of the ingested dose of marionberry Cyg [30].

Cyg are quickly absorbed since they are detected in plasma only a few minutes after gastric administration. Maximal plasma concentrations are usually recorded between 15 and 30 min after administration [7, 31, 32]. Harada *et al.* [9] have reported that maximal plasma concentration was observed 5 min only after administration of acylated Cyg from purple sweet potato tuber.

Whereas some animal studies have shown that Cyg are present in plasma and excreted in urine only under their glycosylated intact forms [6,7], most of the recent ones have demonstrated the presence of several Cy and Cyg metabolites in plasma and urine, such as methylated and/or glucuronidated conjugates [27, 29, 30, 32, 33]. In all these last studies, concentration of native Cyg was higher than concentration of each metabolite.

In rats, a methylated derivative (peonidin 3-glucoside) as well as monoglucuronides of Cy and peonidin were present in urine after blackberry (Cy 3-glucoside) feeding [26, 27]. Acylated Cyg such as Cy 3-malonylglucoside are also recovered in urine as methylated derivatives [29]. Methylated glycosides, monoglucuronides of aglycones as well as monoglucuronides of Cy and peonidin glycosides have been identified in urine from weanling pigs after consumption of various berries [30, 33]. These authors have underlined that the sugar moiety may influence the absorption and metabolism of Cyg. Indeed, they have reported that formation of glucuronidated conjugates of Cy occurred with various monoglycosides (galactoside, arabinoside, xyloside, and glucoside) but the di- or triglycoside forms of Cy were not metabolized to glucuronidated forms. Moreover, in pigs, Cy monoglycosides were metabolized via methylation and glucuronidation whereas delphinidin glycosides were not metabolized to any measurable extent [33].

Cy derivatives present in plasma are mainly native Cyg as well as methylated and/or glucuronidated derivatives. Moreover, low amounts of aglycones (Cy and its methylated derivative peonidin) have been detected in plasma from rats fed a blackberry anthocyanin enriched-diet [27]. Aglycones are very unstable at physiological pH and could thus rapidly degrade. These compounds could correspond to intermediary metabolic forms before enzymatic conversion, *i.e.* glucuronidation.

The stomach seems to play an essential role in Cyg absorption. The acidity of the gastric content should

constitute a favourable environment for these molecules and the rapid appearance of Cyg in plasma could result in part from absorption through the gastric wall. An high proportion of Cy monoglycoside (≈25%) was rapidly absorbed from this organ after direct administration into the rat stomach [34]. This compound was absorbed from the gastric wall without modification [34]. An organic anion carrier, bilitranslocase, expressed in the gastric epithelium, could be involved in the absorption of Cyg at the gastric level [35].

An *in vitro* study using mice intestinal segments has shown that Cy 3-glucoside absorption differed according to the intestinal segments, with the highest absorption occurring in the jejunum, minor absorption occurring in the duodenum and practically no absorption occurring in the ileum or colon [36]. Using an *in situ* intestinal perfusion model, Talavéra *et al.* have shown that Cyg were efficiently absorbed from the small intestine (from 13 to 22% of the perfused dose) [38]. Intestinal absorption was influenced by the glycosidic moiety and Cy 3-glucoside was more absorbed than Cy 3-galactoside or Cy 3-rutinoside [37].

Cyg are mainly absorbed from the small intestine under their glycosidic forms. Once absorbed through the intestinal barrier, they are not excreted back under conjugated forms in the intestinal lumen as was reported for some flavonoids [37, 38]. Cy 3-glucoside is partly hydrolyzed by intestinal β-glucosidases as reflected by the presence of the aglycone (Cy) in the jejunum [28, 39]. Cyg metabolites such as methylated glycosides and Cy glucuroconjugated forms have been identified in the jejunum and plasma [27, 32]. Cy glucuroconjugates could be formed in the intestinal tract as suggested by Ichiyanagi *et al.* after comparison of plasma Cyg metabolites following oral or intravenous Cyg administration [32].

Stability of Cyg within the gastrointestinal tract may be a limiting factor for their absorption. For the same aglycone, the sugar moiety is an important factor in determining their concentration in different segments of the gastrointestinal tract [39]. The environment all along the gastrointestinal tract is critical to the stability and may further determine the forms that exist in the intestine and that are absorbed and/or metabolized. Clearly, the glycosidic moiety has major effects on the intestinal absorption and/or metabolism of Cyg [37, 39].

So, a high amount of Cyg is absorbed from both the stomach and small intestine whereas only low concentrations are detected in plasma. This raises the question of Cyg tissue distribution and their possible transformation after absorption. Cy and Cyg chemistry is complex and a large part of absorbed Cy and Cyg could thus be metabolized to uncoloured forms, thereby escaping detection under normal conditions [37].

Metabolism by the intestinal microflora is also one important factor controlling the bioavailability. A few studies have evaluated microflora Cyg degradation *in vitro*. Keppler and Humpf [40] have thus shown that Cyg are hydrolyzed extensively by the intestinal microflora depending on the sugar moiety. After cleavage of the protective 3-glycosidic moiety, the released Cy is very unstable under physiological conditions and could then be further metabolized by the bacteria or degraded by a chemical reaction without the action of bacteria to phenolic acids and aldehydes [40-42]. These metabolites could then be absorbed from the colon and reach the blood stream.

The liver is a major site of enzymatic conversion, particularly methylation and glucuronidation. After blackberry Cyg feeding to rats, native Cyg, methylated glycosides as well as traces of aglycone monoglucuronides are present in the liver [27]. Methylated forms are the main Cyg metabolites recovered in this organ [27, 31]. They could partly result from hepatic methylation by catechol-O-methyltransferase (COMT). The site of methylation differs according to the B-ring structure. Indeed, Cyg methylation mainly occurs at the 3'-hydroxyl moiety [27, 30, 32] but some authors have identified a 4'-*O*-methylated form in plasma or urine [30, 32]. Moreover, methylated derivatives are also the main Cyg metabolites in the bile [37]. These metabolites may thus be excreted from the liver directly into bile. The liver seems thus to be the main organ responsible for methylation of Cy derivatives whereas glucuronidation would mainly occur in the intestine [32].

The kidney is the last organ where Cy derivatives transit before urinary elimination. Methylated forms have been detected in the kidney [27, 31, 38]. Moreover, monoglucuronides of aglycones have been identified in this organ. They could partly result from the action of an UDP-glucuronosyltransferase, since such an enzyme is expressed in this organ [43].

Cyg have been detected in some potential target organs such as brain and eye. Indeed, Cyg can target the brain of rats following consumption of an anthocyanin-enriched diet [27, 44]. They have been localized in brain regions that mediate cognitive behaviour (cortex, hippocampus) [45]. Cyg are thus able to cross the blood-brain barrier in accordance with an *in vitro* study showing that brain endothelial cell lines took up Cu 3-rutinoside [46]. However, the mechanisms of entry into the central nervous system remain unknown. Moreover, Cyg are absorbed and distributed as intact forms into ocular tissues. They could thus pass through the blood-aqueous barrier and blood-retinal barrier in both rats and rabbits [47].

Antioxidant activity

The utility of Cy and Cyg in the treatment of pathologies where free radical production plays a key role has been suggested by numerous researches. Particularly, Cy 3-glucoside, alone or together with other Cy 3-glycosides and with the aglicone form, has been widely investigated with the aim to establish its antioxidant activity in different experimental conditions. Acquaviva *et al.* [48] showed that Cy 3-glucoside and Cy had a protective effect on DNA cleavage, a dose-dependent free radical scavenging

activity and significant inhibition of xanthine oxidase activity. Cy 3-glucoside, which has been identified as the main anthocyanin of blackberry *(Rubus species)* juice, has been showed to be a scavenger of peroxynitrite and to be able to exert a protective effect against *in vitro* endothelial dysfunction and vascular failure induced by peroxynitrite tested on human umbilical vein endothelial cells (HUVEC) [49]. Duthie *et al.* [50] investigated the consequence both of vitamin E deficiency on oxidative damage to DNA and lipids and the cytoprotective effect of nutritionally relevant levels of Cy 3-glucoside (100 mg/kg; 12 weeks) both *in vivo* in rats and *in vitro* in human colonocytes. The authors found that whereas Cy 3-glucoside protected against oxidative DNA damage *in vitro*, it did not alter lipid peroxidation or DNA damage in rats. Guerra *et al.* [51] showed the ability of Cy 3-glucoside to reduce the production of reactive oxygen species (ROS), the inhibition of protein and DNA synthesis and the apoptosis caused by aflatoxin B1 and ochratoxin A in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). The antioxidant activity exerted in a liposomal membrane system by different Cyg (arabinoside, rutinoside, galactoside and glucoside) has been found [52] to be higher than that of trolox in the case of Fe(II)-induced liposome oxidation and to be comparable with the action of trolox (a water-soluble tocopherol derivative) in the case of UV- and AAPH (2,2'-azobis[2-amidinopropane] dihydrochloride)-induced liposome membrane oxidation. Cy 3-glucoside and Cy 3-rutinoside, were quantified by Lichtenthaler *et al.* [53] as the two major anthocyanins of eleven commercial and non-commercial samples of *Euterpe oleracea Mart.* (acai) fruit pulp that showed good antioxidant capacities against peroxyl, peroxynitrite and hydroxyl radicals.

The inhibitory effects of lipid peroxidation exerted by Cy 3-glucoside, Cy and Cy 3-galactoside have been showed and quantificated by Adhikari *et al.* [54] who also found a good effect respect to commercial anti-oxidants butylated hydoxyanisole, butylated hydroxytoluene, and tert-butylhydroxyquinone.

Cy 3-glucoside has been showed to be able to modulate hepatic stellate cells proliferation and type I collagen synthesis induced by a ferric nitrilotriacetate complex as pro-oxidant agent, thus suggesting a potential role for this antioxidant compound in the prevention of fibrosis in chronic liver diseases [55]. In a test performed *in vitro* on in human fibroblasts by Russo *et al.* Cy 3-glucoside significantly reduced free radical species production and prevented genomic DNA damage due to ochratoxin A (OTA), a mycotoxin produced by *Aspergillus ochraceus* and other moulds with carcinogenic, teratogenic and nephrotoxic properties in both humans and farm animals [56].

As regards other Cyg, Cy 3-galactoside showed a stronger activity respect to that of other flavonoids as well as vitamin E or Trolox from cranberries' extracts in two antioxidant assays consisting in the evaluation of 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity and in the ability to inhibit low-density lipoprotein oxidation *in vitro* [57]. Cy 3-malonylglucoside, identified as one of the main phenolic compounds from red leaf lettuce *(Lactuca sativa L.)* [58], showed a marked alkylperoxyl radical radical scavenging activity. Different Cyg from Cichorium genus vegetables showed antioxidant activity consisting in trapping peroxyl radicals [59]. Cy 3-O-(2-O-(6-O-(E)-caffeoyl-beta-D-glucopyranocyl)-beta-D-glucopyranoide)-5-O-beta-D-glucopyranoside from *Ipomoera batatas cultivar Ayamurasaki*, finally, has been proved to have a strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity *in vitro* as well as *in vivo* [60].

Cy also showed its antioxidant activity. It was, infact, the stronger superoxide radical scavenger among the polyphenols of different varieties of plums [61] and exerted a protective effect against the toxicity induced by linoleic acid hydroperoxide (LOOH) in cultured human fetal lung fibroblasts, TIG-7 [62]. Similarly, Cy inhibited malonaldehyde formation in oxidized calf thymus DNA oxidized by Fenton's reagent [63].

Lazze *et al.* [64] showed that Cy was able to protect rat smooth muscle and hepatoma cell lines against cytotoxicity, DNA SSB formation and lipid peroxidation induced by tert-butyl-hydroperoxide (TBHP) whereas its glycoside and rutinoside derivatives did not work.

The antioxidant activity exerted by Cy and by some glycosidic forms has been confirmed in three lipid-containing models (human low-density lipoprotein – LDL – and bulk and emulsified methyl linoleate) by Kahkonen *et al.* [65] who also linked the differences in the antioxidant power with the different glycosylation patterns. Cy isolated from extracts of bilberry, finally, exhibited a protective effect against ocular diseases by an antioxidant activity against the photooxidation of A2E, an autofluorescent pigment that accumulates in retinal pigment epithelial cells with age and in some retinal disorders such as against membrane permeabilization in retinal pigment epithelial cells [66].

BIOLOGICAL PROPERTIES

Basing on the data review by Galvano *et al.* [4], the results of several *in vitro* and *in vivo* reports, suggesting that Cy and Cyg have different biological properties that render them as natural compounds with possible beneficial effects in various human, are presented in *Table 2*.

Anticarcinogenic activity

Cyg and their derivates have been widely tested with the aim to establish their anticarcinogenic effect. Both *in vitro* and *in vitro* tests have been performed on different experimental conditions and suggested that the anticancer activity may be due to their antiproliferative, pro-oxidant and apoptotic effects as well as to their capability to regulate gene ex-

Table 2 | *Biological properties of cyanidins*

Properties	References
Free radical scavenging activity	[48, 49, 58, 60, 61]
Protection against oxidative DNA damage	[48, 50, 56, 64]
Protective effect against oxidative endothelial dysfunction and vascular failure	[49]
Reduction of reactive oxygen species (ROS) production/activity	[51, 56, 53, 48, 59, 64]
Prevention of lipid peroxidation	[52, 54, 57, 62, 64, 65]
Inhibition of the apoptosis caused by aflatoxin B1 and ochratoxin A in a human hepatoma (Hep G2) colonic adenocarcinoma (CaCo-2)cell lines	[51]
Prevention of fibrosis in chronic liver diseases	[55]
Protection towards calf timus DNA against oxidative damage	[63]
Protection against human ocular diseases	[66]
Inhibitory effects against human cancer cell lines	[67-80]
Modulation of human DNA topoisomerases I and II activity in the prevention of DNA damage	[81]
Amelioration of endothelial dysfunction, vasoprotective effects and prevention of the NO-mediated inflammatory diseases	[84-86] [87-92]
Prevention of inflammation	[54, 93-97]
Anti-obesity and anti -diabetes effects	[98-102]
Alpha-glucosidase inhibitory activities	[103, 104]
Regulation of glucose/glycogen homeostasis	[105]
Skin photoprotective effetcts	[106, 107]
Antineurodegenerative activity	[110, 111]
Gastroprotective effects	[112]
Stimulatory effect of on the regeneration of rhodopsin	[113]
Dietary effects (sparing vitamin E in rats)	[114, 115]

pression thus suggesting a possible role in the strategies for different forms of cancer treatment.

Cy 3-glucoside, particularly, have been often tested. Cy 3-glucoside from black bean seed coat displayed strong growth inhibitory effects against human leukemia Molt 4B cells resulting from the induction of apoptosis [67]. Serafino *et al.* [68] showed that the treatment with Cy 3-glucoside was able to revert the human melanoma cells from the proliferating to the differentiated state, thought a strong increase in dendrite outgrowth accompanied with a remodelling of the microtubular network, a dramatic increase of focal adhesion and an increased expression of "brain specific" cytoskeletal components.

Ding *et al.* [69] recently demonstrated that in cultured JB6 cells Cy 3-glucoside inhibited tumour promoter-induced carcinogenesis, reduced the size of A549 tumour xenograft growth and significantly inhibited metastasis in nude mice.

Cy 3-glucoside from *Oryza sativa L.* indica had been showed to be able to induce apoptosis and inhibit tumour cell growth on different cell lines *in vitro* and to suppress lung carcinoma cells growth *in vivo* [70]. When tested on mouse epidermal JB6 Cl 41 cells, Cy 3-glucoside, together with Cy 3-O-(2(G)-xylosyl-rutinoside), and Cy 3-O-rutinoside from freeze-dried black raspberries, clearly inhibited tumor induction by N-nitrosomethylbenzylamine [71].

Interestingly, Cy 3-glucoside, showed a dose-dependent growth inhibition against breast (MCF-7), colon (HCT-116), stomach (AGS), central nervous system (CNS), and lung (NCI-H460) tumour cells [72]. Cy 3-glucoside together with Cy 3-rutinoside from mulberry *(Morus alba L.)* exerted a dose-dependent inhibitory effect on the migration and invasion, of highly metastatic A549 human lung carcinoma cells in absence of cytotoxicity [73]. The potentially chemopreventive mechanisms exerted by Cy 3-glucoside has been investigated by the research team of Fimognari and co-workers who concluded that: Cy 3-glucoside was able to induce apoptosis on transformed and normal lymphocytes T cells by an increase of p53 and bax proteins [74, 75] such as on Jurkat and HL-60 leukemia cell lines [76]; Cy 3-glucoside-induced apoptosis and cytodifferentiation are two distinct events [76, 77].

The aglicone form, Cy, has been also investigated by different authors.

Antiproliferation effects of Cy, through the induction of apoptosis, has been recordered by Yeh *et al.* [78] in human hepatoma cell lines HepG(2) and Hep3B. Similarly, at 200 μg/ml, it inhibited the breast cancer cell line MCF-7 growth at 47% [79] and mediated cytotoxicity against U937 human monocytic leukemia cells by the growth inhibition (arrest of G(2)/M phase) and by the induction of apoptosis when administered at 60 μg/ml concentration [80].

In an *in vitro* test on HT29 cells Habermeyer *et al.* [81] observed that Cy modulated the activity of human DNA topoisomerases I and II, nuclear enzymes interacting with DNA in order to prevent its damage, such as the affinity of Cy to double-stranded DNA.

In contrast with the previous data, some authors found that Cy did not inhibit proliferation of different human cancer cell lines: human uterine carcinoma and colon adenocarcinoma cells [82], MCF-7 (breast), SF-268 (central nervous system, CNS), HCT-116 (colon), and NCI-H460 (lung) when tested at 100-μM concentrations [83].

Vasoprotective effects

Cyg and their derivates can have important implications for the prevention of the NO-mediated inflammatory diseases. Cy 3-glucoside exerts a protective effect against peroxynitrite-induced endothelial dysfunction and vascular failure [49] acting as efficacious scavenger of peroxynitrite a pro-oxidant agent that forms by reaction of NO with superoxide anion (O2-). However, its ability is not limited to the antioxidant activity but also results in the regulation of enzymes involved in the NO activity. Indeed, the reduction of the levels of inducible nitric oxide synthase (iNOS) expression has been recorded in different experiments: Cy 3-glucoside and Cy 3-O-rhamnoside from the palm *Euterpe oleracea*, tipically growing in Brasil, have been tested by Matheus *et al.* [84]; Cy 3-glucoside from blackberry extract has been tested in J774 cells [85]; Cy 3-glucoside orally administered in rats suppressed the zymosan-induced inflammatory response in the peritoneal exudate cells [86].

The amelioration of endothelial dysfunction and the vasoprotective effects exerted by the regulation of the vascular tension regulator endothelial NO synthase (eNOS), a protective enzyme in the cardiovascular system, has been recorded in different conditions: Cy showed its vasoprotective effect in an *in vitro* experiment performed on cultured human umbilical vein endothelial cells (HUVECs) [87]; Cy from red wine increased human eNOS in human EA.hy 926 endothelial cells [88]; Cy 3-glucoside upregulated eNOS production in an *in vitro* test on bovine artery endothelial cells (BAECs), escalated NO production by the phosphorylation of Src and extracellular signal-regulated kinase 1/2 (ERK1/2) [89] and enhanced its activity by regulating its phosphorylation [90].

A recent test on endothelial cells, showed that inhibitory effect of Cy on TNF-alpha-induced apoptosis involved multiple pathways, such as eNOS and thioredoxin expression and Akt activation [91]. In a very recent study on human endothelial cells Sorrenti *et al.* [92], besides confirming that Cy 3-glucoside upregulated e-NOS, also demonstrated that Cy 3-glucoside conferred an additional cytoprotective effect consisting in the induction of the stress protein heme oxygenase-1 (HO-1), that exerts an important cellular protective mechanism against oxidative injury.

Anti-inflammatory effects

Different experimenteal tests estabilished the anti-inflammatory effects and the therapeutic efficacy in experimental models of inflammation exerted by Cyg and their derivates. Cy from berries has been recordered as a potential candidate for the alleviation of arthritis by He *et al.* [93] who showed its anti-inflammatory effects in an *in vivo* test performed by induced arthritis on rats. The authors also suggested the involvement of an antioxidant mechanism by the improvent of the total antioxidative capacity and of the scavenge the free radicals. Cy 3-glucoside from blackberry extract had a dose-dependent therapeutic efficacy in an experimental model of lung inflammation induced by carrageenan in rats [94]. The inhibitory effects of cyclo-oxygenase (COX)-1 and -2 enzymes has been showed and quantificated by Adhikari *et al.* [54] who observed the strong inhibition of Cy 3-galactoside, Cy 3-glucoside and Cy respect to some positive controls: aspirin, Celebrex and Vioxx thus confirming the results found by Seeram *et al.* [95] who compared Cy activity with that of some commercial anti-inflammatory drugs (ibuprofen, naproxen, Vioxx and Celebrex). The molecular basis of COX-2 inhibition exerted by Cy has been related to its ortho-dihydroxyphenyl structure on the B-ring [96]. Another evidence on the efficacy in inhibition of COX-1 and COX-2 enzimes by Cy 3-glucoside, alone and in combination with other natural pigments, has been provided by Reddy *et al.* [97].

Anti-obesity and anti-diabetes effects

The potency of therapeutic implications of Cy 3-glucoside for preventing obesity and diabetes has been investigated by Tsuda *et al.* who assessed the potential role of Cy 3-glucoside-rich purple corn colour (PCC) in prevention of obesity and in the amelioration of diabetes. They observed that dietary PCC significantly suppressed the high fat diet-induced increase in body weight gain, white and brown adipose tissue weights and hyperglycemia, hyperinsulinemia and hyperleptinemia [98]. In some studies performed with the aim to investigate the gene expression profiles in human and rat adipocytes treated with anthocyanins, the some researchers found that Cy 3-glucoside-treated rat [99] and human [100] adipocytes enhanced adipocytokine (adiponectin and leptin) secretion and up-regulated the adipocyte specific gene expression but also that isolated rat adipocytes treated with Cy 3-glucoside and Cy demonstrated an up-regulation of hormone sensitive lipase and the enhancement of the lipolytic activity [101].

Cy 3-glucoside, besides, stimulated *in vitro* insulin secretion from rodent pancreatic beta-cells (INS-1 832/13) at 4 and 10 mM glucose concentrations [102].

As regards other Cyg Cy 3-rutinoside has been proposed as a new non-competitive alpha-glucosidase inhibitor because of its ability to inhibit alpha-glucosidase from baker's yeast [103]. Similarly, the alpha-glucosidase inhibitory activities and the antihyperglycemic action of small crimson fruit of *Viburnum dilatatum Thunb.* has been linked to its content in Cy 3-sambubioside (C3S) [104].

Cy also seems to have also a role in regulation of glucose/glycogen homeostasis by the inhibition of

phosphorylated, active form of glycogen phosphorylase (GPa) [105].

OTHER PROPERTIES

Skin photoprotective effects

Two recent *in vitro* studies on human keratinocytes (HaCaT) concluded that Cy 3-glucoside could successfully be employed as a skin photoprotective agent against, respectively, ultraviolet-A [106] and -B [107] (UVA and UVB) radiations.

Tarozzi *et al.* [107] particularly, showed that UVA-induced apoptosis and DNA fragmentation caused by the generation of reactive oxygen species (ROS) has been counteracted by Cy 3-glucoside by the inhibition of hydrogen peroxide (H_2O_2) release after UVA irradiation and by the enhancement of the resistance to the apoptotic effects of both H_2O_2 and the superoxide anion (O2-). The authors also suggested that Cy 3-glucoside protective effects could be attributed to the high membrane levels of incorporation.

Antineurodegenerative activity

Cy derivatives from cherry [108] and Cy 3-glucoside from mulberry fruit extract [109] have been assumed as neuroprotective constituent on the PC12 cells exposed to cell-damaging oxidative stress. Neuroprotective effects against cerebral ischemic damage *in vivo* has also been performed by Kang *et al.* [109] using a mouse-brain-injury model with a transient middle cerebral artery occlusion (MCAO).

Gastroprotective effects

Cy 3-glucoside has been identified as one of the main components of the red pigment fraction of black chokeberry fruit *(Aronia melanocarpa Elliot)* which showed strong gastroprotective effect on acute gastric hemorrhagic lesions in rats caused by the subsequent application of ethanol [110].

Ocular effects

The function of Cy 3-glucoside and Cy 3-rutinoside on visual functions has been suggested by Matsumoto *et al.* [111] who recorded their stimulatory effect of on the regeneration of rhodopsin.

Dietary effects

Frank *et al.* [112] tested the effects of dietary Cy 3-glucoside on plasma and tissue concentrations of tocopherols and lipids in rats founding that it seemed to be capable of sparing vitamin E in healthy, growing rats but also that it had little effect on cholesterol levels. Similar antihyperlipidemic effect in rats fed a high-cholesterol diet has been recently attributed to Cy 3-glucoside [113].

CRITICAL CONSIDERATIONS AND FUTURE STUDIES

As specifically regards the metabolic fate of Cy and Cyg we believe that their absorption and metabolism is still far to be completely elucidated. The first requirement for a dietary compound to have a protective role for human health is that it enters the blood circulation. Cy and Cyg absorption is confirmed by the recovery of known metabolites in plasma and urine. However, in the majority of literature studies authors conclude that Cy and Cyg bioavailability is scarce basing on the recovery of very low amount of known metabolites in urine and/or plasma. We do not agree this conclusion. Indeed, it is a fact that, so far, all of literature studies failed in revealing the metabolic fate of the very large part of ingested Cy and Cyg. Indeed, if Cy and Cyg bioavailability was truly scarce they should be massively found undigested and/or partially metabolized in the faeces, but no studies demonstrated it. Thus, the most logical conclusion should be that, although some metabolites (methylated and/or glucuronidated conjugates) have been described, some other important metabolites may still not have been identified, in relation to the complex chemistry of these compounds. Indeed, Cy and Cyg analysis is usually carried out after conversion of the various chemical forms into coloured flavylium cation by acidification. It could thus be hypothesized that some forms existing at neutral pH would not be converted into the flavylium form or would rapidly degrade into unknown metabolites. These important points may need further human controlled studies. In spite of not definitive conclusions on Cy and Cyg bioavailability, the abundant mass of available data reviewed from literature allows affirming that these dietary compounds confirm a great potential beneficial role in human health. However, researchers are requested of a severe scientific objectivity in evaluating health properties of food ingredients. So we can not avoid to reiterate the critical conclusions of our previous review on Cy and Cyg [4]. It has to be still noted that the majority of studies are conducted in a multitude of *in vitro* experimental models, mostly cell cultures. Cell cultures are an approach of great importance in the preliminary stage of research and are used as an investigative model alternative to expensive and time-consuming clinical studies. However, prudence is necessary in the use and interpretation of *in vitro* experiments on polyphenols. Two are the common flaws of such approach. The first is Cyg do not reach plasma and tissues in their natural forms, but mainly in the metabolized ones (*i.e.* methylated, glucuronidated conjugates and others still to be identified) whose biological activity could be different. The second, lies with the concentrations tested *in vitro*, which are often several orders of magnitude higher than the plasma concentration likely reachable after a normal meal. An analogous inaccuracy is also present in some in both human and animal studies on metabolism and bioavailability, where administered doses were higher than the mean presumable dietary intake. Other uncertainties derive from the fact that the referred studies report data on Cy and Cyg considered both as single chemicals and food extracts. In our opinion any definitive nutritional conclusion

on food compounds can not be done by evaluating it avulse from the food itself. Indeed, a single-molecule approach would be of pharmacological type and would need an amount of further studies even more significant, including exhaustive information on absorption, distribution, metabolism and excretion of Cy and Cyg administered by main possible routes (oral, intraperitoneal, intravenous, intratecal).

In conclusion, with the aim to establish whether these compounds are really capable to influence positively the incidence and progression of many chronic diseases, a great deal of work in several areas is still necessary. This includes i) further studies on Cy and Cyg metabolism in human beings based on whole foods consumption; ii) analysis of factors affecting bioavailability, including interaction with other dietary compounds (*i.e.* other flavonoids); iii) dietary burden and variations within and between populations; iv) epidemiological studies to evaluate the relationship between Cyg-rich food consumption and incidence of given pathologies. The ancient Chinese saying "food is my medicine" has become very popular. As correctly highlighted by Fogliano and Sacchi [114] from the standpoint of molecular nutrition, food cannot be considered as a drug where the active compound is concentrated and formulated. A rigorous application of evidence based medical rules to studies on food could increase the quality of science in this field and would avoid generating false myths among consumers about miraculous foods [114].

However, likely as for no other food compound, the preliminary evidences of potential protective effects of Cy and Cyg embrace a so wide spectrum of human pathologies, there is no doubt on the need of further great attention by researchers at various title involved in nutrition and human health.

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