In vitro bioactivities of dietary anthocyanins and proanthocyanidins: implications for bioavailability, neuroprotection and safety

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[…] one's title […] says very little about how well one's life has been led -- that no matter how much you've done, or how successful you've been, there's always more to do, always more to learn, and always more to achieve.

Barack Obama

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Index

Abbreviations

Aβ	β-amyloid
ABC	ATP-binding cassette
ACE	acetylcholinesterase
AD	Alzheimer's disease
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
ALS	amyotrophic lateral sclerosis
ANOVA	one-way analysis of variance
ara	arabinoside
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCRP	breast cancer resistance protein
bp	base pairs
BSA	bovine serum albumine
bw	body weight
©	copyright
calcein-AM	calcein acetoxymethyl ester
ChT-L	chymotrypsin-like
CNS	central nervous system
CoA	coenzyme A
COX	cyclooxygenase
$cPLA_2$	Ca^{2+}-dependent cytosolic PLA_2
CYP	cytochrome P450
DAD	diode array detection
def	defective
df	degrees of freedom
DFO	desferrioxamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	degree of polymerization
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EC	European Community

EC	effective concentration
ECG	epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
eff	efficacy
EFSA	European Food Safety Authority
EGCG	(-)-epigallocatechin gallate
EGE	ethylene glycol ester
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric-oxide synthase
EPAS	endothelial PAS domain protein
EPO	erythropoietin
ESI	electrospray ionization
FAO	Food and Agriculture Organization
FCS	foetal calf serum
FGFR	fibroblast growth factor receptor
FIH	factor inhibiting HIF
FUFOSE	Functional Food Science in Europe
g	gram
gal	galactoside
glc	glucoside
GLUT	glucose transporter
GSPE	grape seed proanthocyanidin extract
h	hour
HBSS	Hank’s Balanced Salt Solution
3-HC	3-hydroxycinnamic acid
HD	Huntington’s disease
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia inducible factor
HO	heme oxygenase
3-HPA	3-hydroxyphenylacetic acid
4-HPA	4-hydroxyphenylacetic acid
HPLC	high performance liquid chromatography
3-HPP	3-(3-hydroxyphenyl)propionic acid
4-HPP	3-(4-hydroxyphenyl)propionic acid

3-HPV	5-(3-hydroxyphenyl)valeric acid
HRP	horseradish peroxidase
HSP	heat shock protein
[I]	inhibitor concentration
IC	inhibitory concentration
ICAM	Intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of kappa B
IL	interleukin
iNOS	calcium-insensitive nitric oxide synthase
IPCS	International Programme on Chemical Safety
$iPLA_2$	Ca^{2+}-independent cytosolic PLA_2
JECFA	Joint FAO / WHO Expert Committee on Food Additives
kDa	kilodalton
kg	kilogram
K_i	dissociation constant
K_m	Michaelis-Menten constant
LB plot	Lineweaver-Burk plot
LD	lethal dose
LDL	low density lipoprotein
LOX	lipoxygenase
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MDR	multidrug resistance protein
ME	methyl ether
MHC	major histocompatibility complex
min	minute
ml	milliliter
MOCAP	*O*-ethyl-S,S-dipropyl phosphorodithioate
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MRP	multidrug resistance associated protein

MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	mass-to-charge ratio
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NMDA	N-methyl-D-aspartic acid
NOAEL	no-observed-adverse-effect-level
NTB	2-nitro-5-thiobenzoic acid
OD	optical density
p	para
PA	proanthocyanidin
PAF	platelet-activating factor
PAS	Per/ARNT/Sim
PBS	phosphate-buffered saline
pc	procyanidin
PCR	polymerase chain reaction
PD	Parkinson's disease
pd	prodelphinidin
PHD	prolyl 4-hydroxylase
PLA_2	phospholipase A_2
PMA	phorbol-12-myristate-13-acetate
PPXE	phenylpiperazinylyl ether
®	registered trademark
RLU	relative light units
ROS	reactive oxygen species
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
rut	rutinoside
[S]	substrate concentration
SD	standard deviation
SGLT	sodium-glucose linked transporter
SNRI	selective norepinephrine reuptake inhibitor
$sPLA_2$	secretory phospholipase A_2
SRM	single reaction monitoring
SSRI	selective serotonin reuptake inhibitor

Taq	Thermus aquaticus
TCA	tricyclic antidepressant
TDI	tolerable daily intake
TIC	total ion chromatogram
TM	registered trademark
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UDP	uridinyl diphosphate
v	initial velocity
VEGF	vascular endothelial growth factor
VCAM	vascular cell adhesion molecule
VIS	visual
V_{max}	maximum velocity
WHO	World Health Organization

Summary

Over the past decades, awareness has increased of multiple health-promoting effects of diets rich in anthocyanins and proanthocyanidins and, specifically, of these compounds' potential for conferring neuroprotection. The present study compiles evidence obtained *in vitro* that expands our understanding of anthocyanin and proanthocyanidin functionalities at multiple levels.

Firstly, anthocyanin and anthocyanidin bioavailability was addressed using a combination of ATPase assays, dye extrusion assays and vesicular transport assays. This approach highlights the contribution made by efflux transporters MDR1 and BCRP to the absorption of berry polyphenols and to their distribution to target tissues including the central nervous system. All test compounds interacted with the BCRP transporter *in vitro*, seven emerged as potential BCRP substrates and 12 as potential inhibitors of BCRP. Two anthocyanidins, malvidin and petunidin, exhibited bimodal activities, serving as BCRP substrates at low micromolar concentrations and, at higher concentrations, as BCRP inhibitors. Effects on MDR1, in contrast, were weak, as only aglycones exerted mild inhibitory activity in the high micromolar range. Distinct affinities of several anthocyanins and the respective aglycones for BCRP suggest that they may be actively transported out of endothelia. Agents that interfere with BCRP activity are therefore likely to facilitate crossing of the intestinal and blood-brain barriers and to augment anthocyanin bioavailability.

Secondly, novel modes of action were sought to rationalize berry polyphenols' direct modulation of neuronal transmission as opposed to their non-specific antioxidant activities. The candidate effectors include cellular monoamine oxidases (MAO) A and B, hypoxia inducible factor (HIF), the proteasome, and phospholipase A_2 (PLA_2).

Elevated MAO activity has long been implicated in the etiology of depression, anxiety and neurodegenerative illness. MAO inhibiting compounds may thus hold promise in the prevention of behavioral symptoms and cognitive decline. For both MAO isoforms, inhibitory effects of anthocyanins and anthocyanidins are illustrated by IC_{50} values in the low micromolar range whereas proanthocyanidins and phenolic metabolites were less effective inhibitors. Kinetic analyses, performed with cyanidin and cyanidin-3-glucoside, indicated a competitive interaction of cyanidin in terms of MAO A, plus a mixed competitive and non-competitive mode of interaction of cyanidin in terms of MAO B as well as of cyanidin-3-glucoside with respect to both enzyme isoforms. Thus MAO inhibition by anthocyanins and their aglycones *in vitro* lends support to central nervous functionalities of diets rich in berry polyphenols and opens new opportunities in the prevention of neuronal pathologies.

Effects on HIF expression were examined to assess candidate compounds' role in enhancing cellular resistance to oxidative stress. By inducing a dose-dependent increase in HIF expression, delphinidin may initiate a variety of cellular survival processes that are inhibited by free iron. This finding argues in favor of iron-chelating properties as a further means of mediating neuroprotection. Other inducers of HIF expression in neuroblastoma cells included gallic acid, cyanidin and bilberry extract, all of which may modulate HIF-dependent transcription of downstream genes.

Inhibition of chymotrypsin-like proteasome activity by test compounds was investigated in HL-60 cells. By its role in mediating the degradation of misfolded proteins and cell cycle regulatory proteins among others, the proteasome is closely involved in pathways that may increase the risk of neurodegeneration e.g. in Parkinson's disease and in Alzheimer's disease. Based on a chemiluminescent assay, anthocyanins and their aglycones achieved IC_{50} values for inhibition of proteasome activity ranging from 7.8 µM for kaempferidinidin and pelargonidin to 32.4 µM for delphinidin. This adds to our understanding of further cellular effectors that may control anthocyanins' antiinflammatory, antioxidative and neuroprotective activities.

Finally, secretory phospholipase A_2 ($sPLA_2$)-modulating effects of berry polyphenols were studied. The anthocyanidins cyanidin, malvidin, peonidin, petunidin, and delphinidin inhibited $sPLA_2$, achieving K_i values < 18 µM, and thus may meditate neuroprotection by counteracting inflammatory processes, whereas tested glycosides proved to be less effective. These results suggest that anthocyanidins may serve to develop treatments for brain disorders that involve oxidative stress, changes in phospholipid metabolism, accumulation of lipid peroxides, and inflammation, including ischemia, multiple sclerosis, epilepsy, and Alzheimer's disease.

Safety of anthocyanins and related compounds is the third focus of this work. Although health-promoting effects of berry polyphenols advocate dietary supplementation with anthocyanin-rich fruit extracts and functional foods, limited data exist on compounds' pharmacokinetics and on possible adverse effects as a consequence of interactions with xenobiotics and other food ingredients. As cytochrome P450 3A4 (CYP3A4) controls the metabolism of about 60% of all drugs, its inhibition may dramatically affect drug safety. In addition, cytochrome P450 2D6 (CYP2D6) inhibitors may provoke adverse effects and limit the use of psychoactive drugs. Addressing berry constituents' cytochrome P450 inhibitory effects, test polyphenols featured IC_{50} values from 12 µM up to 7,842 µM in terms of CYP3A4. In the order of decreasing effect size, anthocyanidins were followed by anthocyanins, proanthocyanidins, and phenolic acids. For CYP2D6 inhibition, IC_{50} values ranging from 55 µM to > 800 µM were obtained. When compared to earlier data on grapefruit furanocoumarins, cytochrome P450

inhibitory activity of tested anthocyanins and anthocyanidins was shown to be 1,000- to 10,000-fold weaker. To judge by these findings, anthocyanins, anthocyanidins, proanthocyanidins and phenolic acids therefore should pose only a limited risk of food–drug interactions mediated by these cytochrome P450 isoenzymes.

Zusammenfassung

Im Laufe der letzten Jahrzehnte wurde die Vielzahl gesundheitsfördernder Effekte einer Anthocyan- und Proanthocyanidin-reichen Ernährung verstärkt wahrgenommen, insbesondere das Potenzial dieser Substanzen neuroprotektive Wirkungen zu erzielen. Die im Rahmen der vorliegenden Arbeit zusammengetragenen *in vitro*-Befunde belegen dies und erweitern unser Verständnis über die facettenreiche Funktionalität von Anthocyanen und Proanthocyanidinen.

Zunächst wurde mit einer Kombination indirekter und direkter Transporter-Assays die Bioverfügbarkeit von Anthocyanen und Anthocyanidinen thematisiert. Dieser Ansatz betont, dass die Efflux-Transporter MDR1 und BCRP einen wichtigen Beitrag zur Absorption von polyphenolischen Beereninhaltsstoffen und zu deren anschließender Verteilung auf Zielgewebe, einschließlich des Zentralnervensystems, leisten können. Alle Testsubstanzen traten *in vitro* in Wechselwirkung mit dem BCRP-Transporter, wobei sich sieben als potenzielle BCRP-Substrate und 12 als potenzielle Inhibitoren herausstellten. Zwei Anthocyanidine, Malvidin und Petunidin, zeigten bimodale Aktivitäten, indem sie in niedrigen mikromolaren Konzentrationen als BCRP-Substrate dienten und in höheren Konzentrationen als Hemmstoffe. Im Gegensatz dazu waren die Effekte auf den MDR1-Transporter nur gering, wobei lediglich die Aglykone nur schwache hemmende Wirkungen im höheren mikromolaren Konzentrationsbereich zeigten. Die ausgeprägten Affinitäten einiger Anthocyane und Aglykone zum BCRP-Transporter legen nahe, dass diese Verbindungen aktiv aus Endothelien transportiert werden. Somit könnten Substanzen, die mit BCRP wechselwirken aller Voraussicht nach den Transport von Anthocyanen und Anthocyanidinen über die Blut-Hirn-Schranke und die gastrointestinale Barriere begünstigen und somit deren Bioverfügbarkeit steigern.

Der zweite Schwerpunkt der vorliegenden Arbeit lag in der Suche nach neuen Wirkmechanismen, die sich für eine direkte Modulation der neuronalen Signalübertragung durch polyphenolische Beereninhaltsstoffe eignen, im Gegensatz zu bereits bekannten nicht-spezifischen antioxidativen Aktivitäten. Als mögliche Effektoren kommen hier die Monoaminoxidasen (MAO) A und B, der Hypoxie-induzierbare Faktor (HIF), das Proteasom und die Phospholipase A_2 (PLA_2) in Betracht.

Einer erhöhten Monoaminoxidase-Aktivität wird schon seit langem eine Rolle in der Ätiologie depressiver, Angst- und neurodegenerativer Erkrankungen zugeschrieben. Somit könnten Monoaminoxidase-hemmende Stoffe vielversprechende präventive Wirkungen auf krankheitsbedingte Verhaltenssymptome und kognitive Abbauprozesse ausüben. Für beide MAO-Isoformen zeigten Anthocyane und Anthocyanidine

hemmende Wirkungen im niedrigen mikromolaren Bereich, wohingegen sich Proanthocyanidine und phenolische Metabolite als weniger effektive Inhibitoren herausstellten. Mit Cyanidin und Cyanidin-3-glucosid durchgeführte Untersuchungen zur Enzymkinetik gaben Hinweise auf kompetitive Wechselwirkungen von Cyanidin bezüglich MAO A. Gemischt kompetitive und nicht-kompetitive Wechselwirkungen wurden für Cyanidin bezüglich MAO B, sowie für Cyanidin-3-glucosid hinsichtlich beider Isoenzme ermittelt. Somit befürworten diese MAO-hemmenden Eigenschaften von Anthocyanen und deren Aglykonen eine Ernährung, die reich an polyphenolischen Beereninhaltsstoffen ist, und eröffnen neue Möglichkeiten bei der Prävention neuronaler Erkrankungen.

Zur Beurteilung einer Wirkung von Beereninhaltsstoffen im Hinblick auf die Steigerung der zellulären Widerstandsfähigkeit gegenüber oxidativem Stress wurden ferner Effekte der Testsubstanzen auf die HIF-Expression untersucht. Die Ergebnisse weisen darauf hin, dass Delphinidin aufgrund konzentrationsabhängiger Erhöhung der HIF-Expression eine Reihe zellulärer Überlebensprozesse einleiten könnte, die durch freies Eisen gehemmt werden. Auf diese Weise könnten Anthocyane durch ihre Eisen-chelierenden Fähigkeiten Neuroprotektion vermitteln. Gallussäure, Cyanidin und Heidelbeerextrakt bewirkten ebenfalls eine Induktion der HIF-Expression in Neuroblastom-Zellen und gelten somit als weitere Kandidaten, welche die HIF-abhängige Transkription nachgeschalteter Gene modulieren könnten.

Die Hemmung der Chymotrypsin-ähnlichen Proteasomaktivität durch die Testsubstanzen wurde in HL-60-Zellen untersucht. Dadurch dass das Proteasom unter anderem eine Rolle bei der Degradierung fehlgefalteter Proteine und regulatorischer Proteine des Zellzyklus spielt, ist dieses Enzym eng eingebunden in Stoffwechselwege die möglicherweise das Risiko neurodegenerativer Erkrankungen erhöhen, so beispielsweise bei Morbus Parkinson oder Alzheimer. In einem Chemilumineszenz-Assay erreichten Anthocyane und deren Aglykone IC_{50}-Werte zwischen 7,8 µM (Kaempferidinidin und Pelargonidin) und 32,4 µM (Delphinidin). Diese Resultate tragen zu unserem Verständnis weiterer möglicher zellulärer Effektoren bei, die antiinflammatorische, antioxidative und neuroprotektive Eigenschaften von Anthocyanen vermitteln.

Abschließend wurden die modulierenden Effekte polyphenolischer Beereninhaltsstoffe auf die sekretorische Phospholipase A_2 ($sPLA_2$) studiert. Die Anthocyanidine Cyanidin, Malvidin, Peonidin, Petunidin und Delphinidin hemmten die $sPLA_2$ mit K_i-Werten < 18 µM und könnten neuroprotektiv wirken, indem sie inflammatorischen Prozessen entgegensteuern. Die geprüften Glykoside erwiesen sich als weniger effektiv. Diese Ergebnisse deuten darauf hin, dass Anthocyanidine Einsatz finden könnten bei der

Entwicklung von Behandlungsmöglichkeiten für Gehirnerkrankungen, die einhergehen mit oxidativem Stress, Veränderungen im Phospholipid-Metabolismus, Akkumulation von Lipidperoxiden und Entzündungen, wie Ischemie, Multiple Sklerose, Epilepsie und Alzheimer.

Den dritten Schwerpunkt dieser Arbeit bildete der Sicherheitsaspekt von Anthocyanen und strukturverwandten Verbindungen. Obwohl die gesundheitsfördernden Eigenschaften von polyphenolischen Beereninhaltsstoffen für eine Nahrungsergänzung mit Anthocyan-reichen Fruchtextrakten und funktionellen Lebensmitteln sprechen, sind nur begrenzte Daten verfügbar über die Pharmakokinetik dieser Substanzen und über mögliche Beeinträchtigungen die als Konsequenz von Wechselwirkungen mit Xenobiotika oder anderen Lebensmittelinhaltsstoffen auftreten könnten. Da das Cytochrom P450 3A4 (CYP3A4) den Metabolismus etwa 60% aller Medikamente kontrolliert, könnte dessen Hemmung die Sicherheit von Arzneimitteln dramatisch gefährden. Zudem könnten Inhibitoren des Cytochrom P450 2D6 (CYP2D6) negative Effekte hervorrufen und die Anwendung psychoaktiver Arzneimittel einschränken. Die durchgeführte Untersuchung möglicher hemmender Effekte polyphenolischer Beereninhaltsstoffe auf Cytochrom P450-Enzyme ergab IC_{50}-Werte zischen 12 µM und 7842 µM hinsichtlich CYP3A4. Dabei nahm die Effektstärke von den Anthocyanidinen über die Anthocyane und Proanthocyanidine bis zu den phenolische Säuren ab. Bezüglich der Hemmung des CYP2D6-Isoenzyms lagen die IC_{50}-Werte zwischen 55 µM und > 800 µM. Im Vergleich mit Daten aus früheren Studien über Furanocumarine aus Grapefruit waren die hemmenden Eigenschaften der geprüften Anthocyane und Anthocyanidine auf Cyrochrom P450-Enzyme 1000- bis 10000-mal schwächer ausgeprägt. Diesen Ergebnissen nach zu urteilen, stellen Anthocyane, Anthocyanidine, Proanthocyanidine und phenolische Säuren somit nur ein eingeschränktes Risiko für Nahrungsmittel-Arzneimittel-Interaktionen dar, die über Cytochrom P450-Enzyme vermittelt werden.

1 Introduction

The growing burden of chronic diseases related to aging and neurodegeneration poses a major challenge to nutritional strategies for prevention. One such strategy may consist in increasing the consumption of plant-derived foods that confer a variety of health benefits [1-3], including a reduction in risk for developing Alzheimer's disease [4]. Such benefits have been associated, at least in part, to the occurrence of secondary plant metabolites (phytochemicals), in particular polyphenols, which exert a wide range of biological activities. While many phytochemicals lack the potency of synthetic pharmaceutical drugs, they may still possess long-term physiological effects when ingested regularly as part of the diet. Some of the most popular nutritional supplements also contain polyphenols such as anthocyanins, proanthocyanidins, flavonols, stilbenes, hydroxycinnamates, coumarins, ellagic acid and ellagitannins, isoflavones, lignans, etc. [5]. Of these, berry constituents as anthocyanins and proanthocyanidins have emerged as powerful neuroprotectants over the last decade [6-9].

However, limited data exist on anthocyanin bioavailability. Especially information on central nervous system bioavailability is limited and investigations with respect to anthocyanin transport across the blood-brain barrier are scarce.

Among the multilevel bioactivities of anthocyanins, their aglycones and phenolic metabolites count neuroprotection and neurorescue mechanisms. Specifically, these compounds may reduce oxidative stress and exert antiinflammatory effects by modifying signal transduction and, ultimately, central nervous plasticity [10].

Although results from *in vitro* and animal studies advocate supplementation with anthocyanin-rich fruit extracts in the light of multiple health-promoting effects, limited data exist on anthocyanin metabolism and on possible adverse effects as a consequence of food–drug interactions. As both foods and nutritional supplements may induce or inhibit drug-metabolizing enzymes [11,12], more information on possible interactions is essential to better estimate the safety of dietary anthocyanins and related compounds.

The present thesis focuses on berry polyphenols' bioavailability, neuroprotective mechanisms and safety. Specifically, *in vitro* interactions with ABC transporters, effects on activities of monoamine oxidases A and B, proteasome and phospholipase A_2, expression of hypoxia inducible factor and CYP3A4 and CYP2D6 activities are addressed.

On the grounds of positive effects of grapes, strawberries and bilberries in rodent studies of neuroprotection [13-19], these fruits' major polyphenolic constituents, namely

anthocyanins and proanthocyanidins, were focused in the present thesis. Moreover, test compounds were selected so as to include structurally diverse and yet common representatives abundant in dietary sources, such as malvidin-glucoside (malvidin-glc) from red grapes and cyanidin-glc from elderberries and blackberries [20-22]. Finally, phenolic acids were examined as potential *in vivo* anthocyanin metabolites [23,24].

2 State of knowledge

2.1 The family of flavonoids

Plant polyphenols comprise a large variety of compounds that may be grouped by their carbon skeleton: Major classes include phenolic acids, flavonoids and the less common stilbenes and lignans [25].

Of these, flavonoids are the most abundant polyphenols in our diets [26]. They are constituents of most vegetables and fruits, various seeds, nuts, grains, spices, tea, and medicinal plants [27]. Flavonoids comprise approximately 9,000 naturally occurring compounds, most of which are found as glycosylated derivates in plants [28]. Chemically, the term “flavonoid” is generally used to describe a broad collection of natural products that include a C6-C3-C6 carbon framework. More specifically, they share a phenylbenzopyran functionality, consisting of two aromatic rings A and B and a heterocyclic C ring [29]. Except for isoflavonoids and neoflavonoids, the flavan (2,3-dihydro-2-phenylbenzopyran) represents the basic flavonoid structure (figure 2-1).

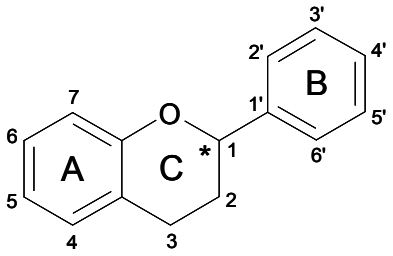

Figure 2-1 Basis flavan structure.

Based on the degree of oxidation and saturation of the heterocyclic C-ring [26], flavonoids may be further devided into the six subclasses, i.e. flavanones, flavones, flavonols, isoflavones, flavan-3-ols and anthocyanins [30]. Varying patterns of hydroxylation, methylation, glycosylation and acylation with phenolic acids plus the occurrence of stereoisomers [25] and polymers [31] allow for significant structural diversity of flavonoids.

Flavonoids are synthesized via the phenylpropanoid metabolic pathway. In the beginning, p-coumaroyl-CoA is produced from the amino acid phenylalanine and malony-CoA is generated during carbohydrate metabolism. Both components are merged to yield a chalcone, the flavonoid backbone. Conjugate ring-closure of chalcones leads to the class of flavonoids. Following a series of enzymatic modifications, further subclasses of flavonoids are formed (figure 2-2) [30,32-36].

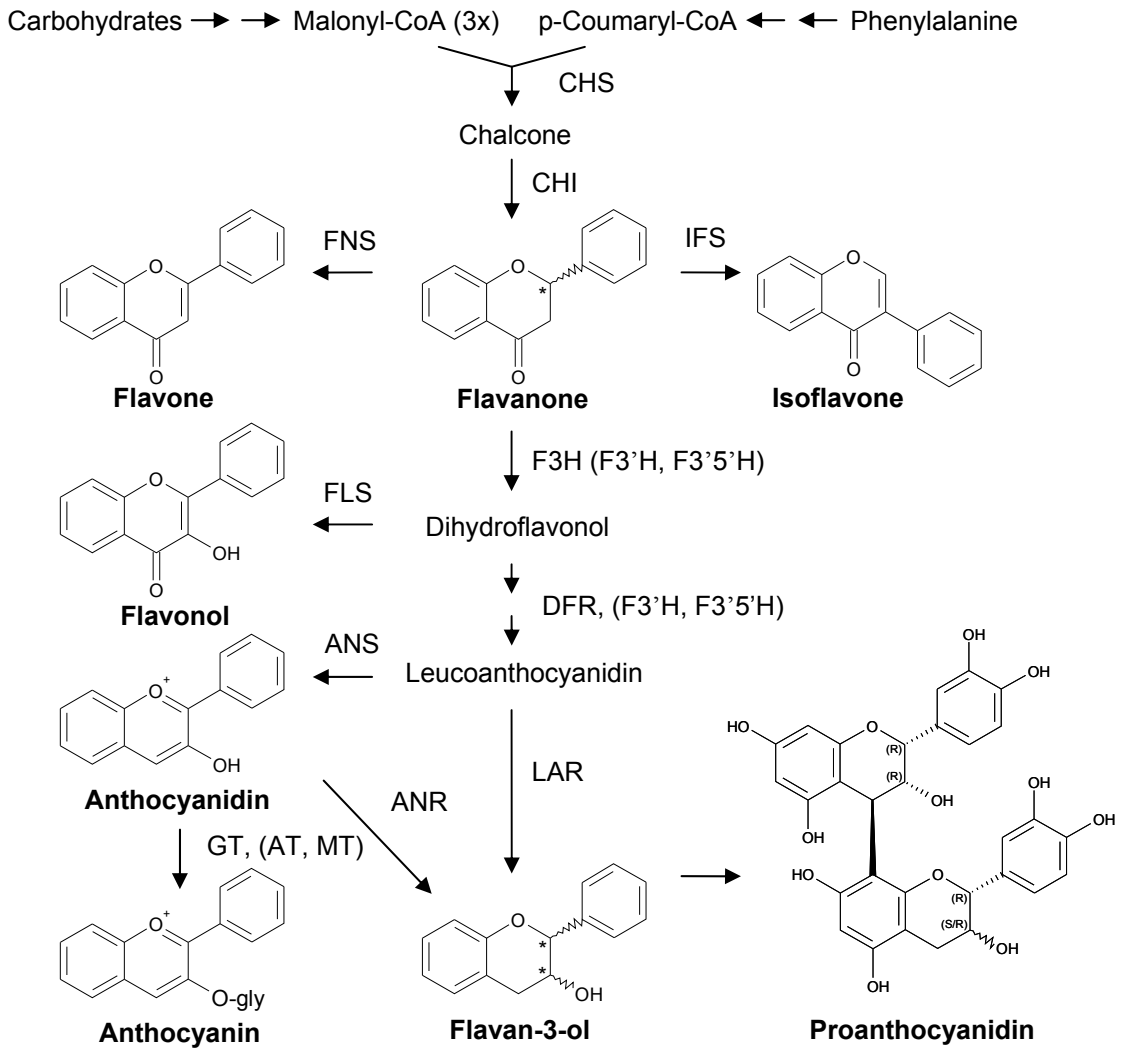

Figure 2-2 Pathways and basic structures involved in flavonoid biosynthesis: For proanthocyanidins, procyanidins B1 (2,3-cis configuration of lower flavan-3-ol unit) and B2 (2,3-trans configuration of lower flavan-3-ol unit) are shown exemplarily. ANR, anthocyanidin reductase; ; ANS, anthocyanidin synthase; AT, acyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; CoA, coenzyme A; DFR, dihydroflavonol 4-reductase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-dihydroxylase; FLS, flavonol synthase; FNS, flavone synthase; GT, glycosyl transferase; IFS, isoflavone synthase; LAR, leucoanthocyanidin 4-reductase; MT, methyltransferase, * chiral centers [30,32-36].

Within the great structural variety of flavonoids, the current work focused on two subclasses of flavonoids, namely anthocyanins and proanthocyanidins.

The term anthocyanin is derived from "anthos" and "kyanos", Greek for "flower" and "blue", respectively, and was first introduced by Ludwig C. Marquart in 1835 [37]. Today, over 500 different anthocyanins have been isolated from plants [38]. Anthocyanins confer cyanic colors ranging from salmon pink through red and violet to dark blue to many flowers, fruits and leaves [38]. With respect to dietary sources, anthocyanins are particularly abundant in berries, grapes, and red cabbage among other foodstuffs. When expressed per 100 g fresh weight, approximately 1,480 mg of

anthocyanins may be obtained from chokeberries, 1,380 mg from elderberries, 687 mg from black raspberries [39], 588 mg from bilberries [40], and 322 mg from red cabbage [39].

The average dietary per capita and day consumption of anthocyanins was originally estimated at 180 to 215 mg in Western societies [41]. More recent calculations from U.S. American surveys have concluded to a daily intake of only 12.5 mg [39], but the actual amount has been shown to vary considerably with sociodemographic and life-style factors [42], plus the seasonal availability of anthocyanin-rich fruits and vegetables [39].

Chemically, anthocyanins are water-soluble, glycosylated polyhydroxyl and polymethoxyl derivatives of flavylium salts. The variety of different glycosyl (i.e., mono-, di- and trisaccharides based on different sugars) and acyl constituents (mainly the phenolic acids p-coumaric, caffeic, ferulic and sinapic acids) contributes to the large number of anthocyanins in nature [43], while the aglycone, named anthocyanidin, is limited to only few structural variants, namely delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin [44] (figure 2-3).

Anthocyanidin	**R1**	**R2**	**R3**
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	O-CH_3	OH	O-CH_3
Pelargonidin	H	OH	H
Peonidin	O-CH_3	OH	H
Petunidin	O-CH_3	OH	OH

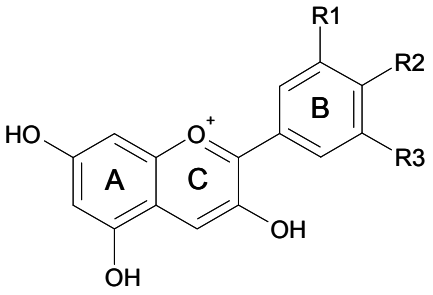

Figure 2-3 Structures of the most common anthocyanidins.

Proanthocyanidins are condensed tannins that are converted to colored anthocyanidins on acid hydrolysis [45]. The wide presence of proanthocyanidins in plants makes them an important part of the human diet, and specifically of fruits, sorghum, beans, nuts, cocoa, and wine [46]. By forming complexes with salivary proteins, proanthocyanidins account for the astringent character of fruits and beverages and for the bitterness of chocolate [47].

Proanthocyanidin (PA) contents of dietary sources per 100 g fresh weight may amount to 330 mg for wild blueberries, 660 mg for chokeberries, 500 mg for hazelnuts, 790 mg for Pinto beans, 1,630 mg for unsweetened chocolate, 1,900 mg for sorghum, 3,500 mg for grape seeds, and 8,100 mg for cinnamon. Red wine and grape juice may yield concentrations of 310 and 524 mg per liter, respectively [46].

Information on the daily intake of oligomeric and polymeric flavan-3-ols is scarce due to the lack of appropriate analytical methodology and commercially available standards for these oligomers. Discrepancies in PA contents have also arisen from differences in cultivars [48], in food processing, in ripening of fruits and vegetables, and in the parts of fruits chosen for analysis. As a result, dietary PA intake may range from several tens to several hundreds of mg per day [47]. With these uncertainties in mind, recent surveys estimate the intake in the US population at 54 mg per day, with tetramers and PAs of higher degrees of polymerization (DPs) amounting to 80% [46].

Chemically, PAs are oligomeric or polymeric flavan-3-ols with elementary units linked by C-C and occasionally C-O-C bonds [49]. They differ structurally according to the number of hydroxyl groups, the stereochemistry of the asymmetric carbons of the heterocycle, the nature of the interflavan bond, the degree of polymerization [48], and acylation and glycosylation patterns [50].

The most common PAs in food are procyanidins (pcs) with a 3',4'-dihydroxy substitution on the B-ring, followed by prodelphinidins (pds) with a 3',4',5'-trihydroxy substitution, which can also occur as mixed pc/pd polymers [51]. The three carbons C2, C3 and C4 of the flavan-3-ol heterocycle are asymmetric and may occur in different configurations. With some very rare exceptions, the configuration of C2 is *R* [48]. The stereochemistry of the C2-C3 linkage may be either *trans* (2*R*, 3*S*) or *cis* (2*R*, 3*R*) as in (+)-(gallo)catechin and (-)-epi(gallo)catechin polymers, respectively. The interflavan bond at C4 is always *trans* with respect to the hydroxy group at C3 [47]. The most common interflavanol linkages are C-C bonds established between the C4 of one flavanol unit and the C8 or C6 of another. The respective PAs are referred to as B-type (dimeric) and C-type (trimeric) PAs [49].

Degrees of polymerization for proanthocyanidins vary markedly. With respect to apple cider, DPs have been reported in the range of 7 to 190 [52] whereas DPs of up to 28 have been described for grape seed extract [53].

Procyanidins B1 and B2 represent C4-C8 linked (-)-epicatechin-(+)-catechin and (-)epicatechin-(-)-epicatechin dimers (figure 2-2). They are considered the main dimeric proanthocyanidins in dietary PA sources [47], with procyanidin B1 occurring in grapes, sorghum and cranberries and procyanidin B2 in apples, cocoa beans and cherries, among others [54].

2.2 Stability, bioavailability and metabolism

2.2.1 Anthocyanins

2.2.1.1 Anthocyanin stability

For anthocyanins' protective action in human health to be fully understood, their stability, bioavailability and bioactive metabolites must be known. Anthocyanins are not stable in aqueous solutions and undergo rapid, pH-dependent, reversible transformations. They may exist in at least 4 different pH-dependent structural isoforms, namely flavylium cations, hemiketals, quinoidal bases, and chalcones, arising at pH values of 1 to 3, 3 to 5, 6 to 8 and 7 to 8, respectively [55].

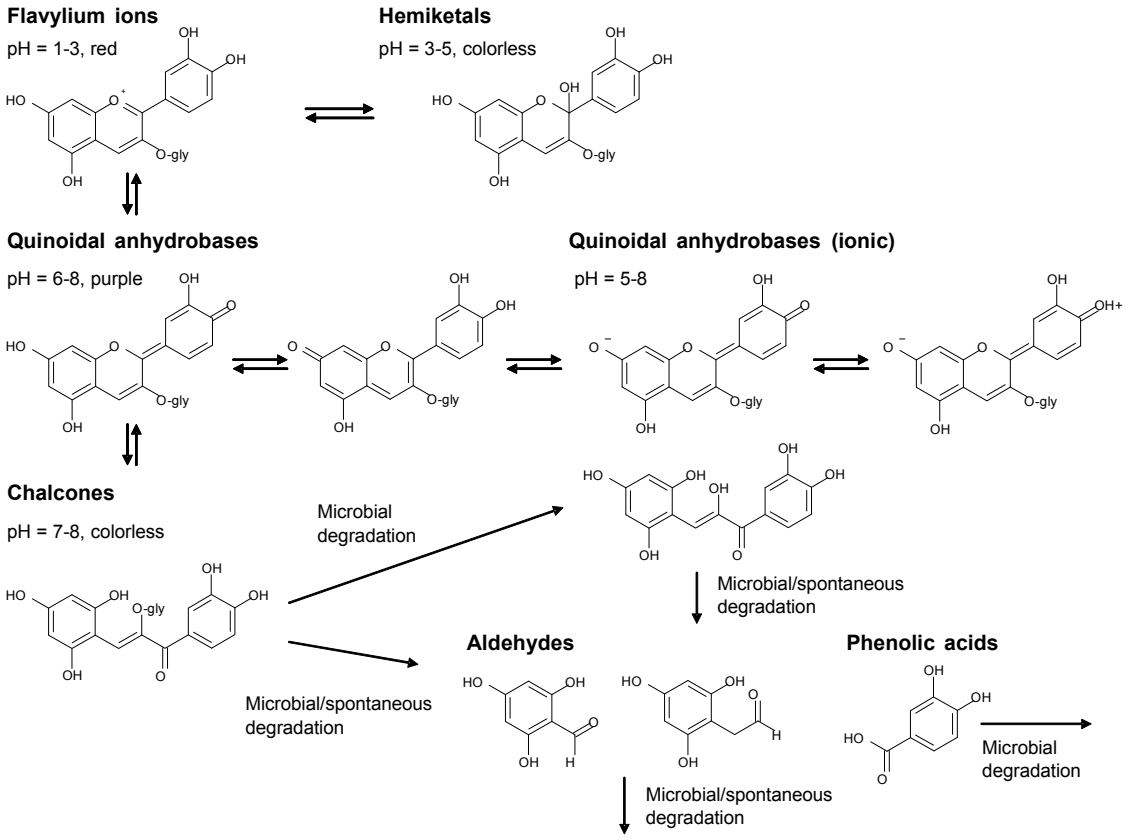

Figure 2-4 Hypothetical pH-dependent structural changes and degradation of cyanidin-glycosides [36,55-62].

Regarding pH-dependent stability of black currant anthocyanins in aqueous solutions, a local minimum stability was detected at pH 3.8 and a rapid decrease in stability at pH > 4.5 [60]. Moreover, upon heating to 95 °C at pH 3.5, anthocyanin half lives of 2 to 3 h were observed [61]. In phosphate buffer and cell culture media at physiological pH and 37 °C, anthocyanins and anthocyanidins were shown to decompose

spontaneously, generating phloroglucinol aldehyde and their corresponding phenolic acids such as gallic acid, syringic acid, vanillic acid and *p*-hydroxybenzoic acid [55,57,59,63]. Glycosides were more stable than aglycones, suggesting that the sugar moiety may prevent degradation of the highly unstable α-diketone, and the number of hydroxyl and methoxy substituents was proportional to the rate of degradation [55,57,59].

Using an *in vitro* model of gastro-intestinal break-down, anthocyanin concentrations did not change after pepsin digestion. Following pancreatic digestion and dialysis, serum available anthocyanins were estimated at 15 to 21% and colon-available anthocyanins at 52 to 67% with respect to frozen cherries [64].
In vitro incubation of anthocyanins with human faecal microflora to simulate microbial and chemical degradation generated corresponding phenolic acids, 3-monoglucoside intermediates for diglucosides [57], plus small amounts of aglycone [56], suggesting microbial degradation via the aglycone [65]. However, generated phenolic acids did not completely account for degenerated parent anthocyanins and phenolic acids were not or only slightly degraded to further metabolites [56,57].
A similar approach using pig caecum microflora confirmed generation of aglycones, phenolic acids, aldehydes and small amounts of aglycones upon anthocyanin incubations. While a low rate of chemical degradation to phenolic acids, but not phloroglucinol aldehyde, was observed for anthocyanins, anthocyanidins exhibited a high rate of chemical degradation generating phenolic acids and phloroglucinol aldehyde. Therefore it cannot be distinguished whether ring fission of anthocyanidins was caused primarily by microbial cleavage or by chemical decay. For chemically quite stable phenolic acids microbial degradation was observed and for phloroglucinol aldehyde chemical and microbial degradation may occur [58].

With respect to animal and human studies administrating complex polyphenol mixtures, phenolic acid metabolites may also derive from metabolism of other flavonoids and ascribing them to anthocyanin degradation is speculative. However, some data on anthocyanin stability *in vivo* can be obtained from animal feeding studies. Cyanidin-glycosides from black raspberry were shown to be relatively stable in the gastric and small intestinal lumens of fasted rats, with 75-79% of administered anthocyanins being recovered from stomach and small intestine 120 min post administration. Cyanidin aglycone was identified in stomach and small intestine contents, suggesting involvement of acidic hydrolysis, brush border membrane enzymes and microbial

activity. Since protocatechuic was not detected, phenolic acids may be promptly degraded or primarily produced in the large intestine [66].

When rats were fed with raspberry juice by gavage, after 1 h 32% and 59% were recovered from stomach and ileum, respectively, after 2 h 86% were found in ileum, after 3 h 40% and 7% were detected in ileum and other parts of the digestive tract, respectively, after 6 h 2% were identified in caecum and colon and after 24 h 1.5% were excreted with faeces. This suggests that significant amounts pass into the large intestine where degradation occurs due to the action of colonic bacteria [67]. In pigs, 42% of administered anthocyanins were recovered in the gastrointestinal tract, primarily in the ileum, caecum and colon [68], with anthocyanin stability during gut passage greatly depending on sugar moieties [69].

2.2.1.2 Anthocyanin bioavailability and metabolism

Bioavailability may be defined as the proportion of a nutrient or bioactive component that is absorbed from the gastrointestinal tract and that reaches systemic circulation, as characterized by plasma concentrations [70]. More generally, bioavailability may be defined as the extent to which a compound becomes available at the site of action [71], and may be further specified in terms of metabolism, excretion, utilisation and efficacy [70].

Basic information on anthocyanin bioavailability has derived from *in vitro* models. Thus it was demonstrated that blueberry and red grape skin anthocyanins can cross a model of the absorptive intestinal epithelium, albeith at an efficacy of only 1 to 4% [72,73]. Ethanol and chronic anthocyanin exposure may increase anthocyanin transport [72]. However, not all studies have been able to confirm transport across the monolayer, implying that additional factors may need to be considered, e.g. cellular metabolism and translocation across the basolateral membrane [74]. Comparatively high rates of anthocyanin absorption were observed in mouse jejunal tissues and in duodenum, but no uptake from ileum or colon tissues was detected [75].

Results from *in vivo* studies are less conclusive owing to marked interindividual variability.

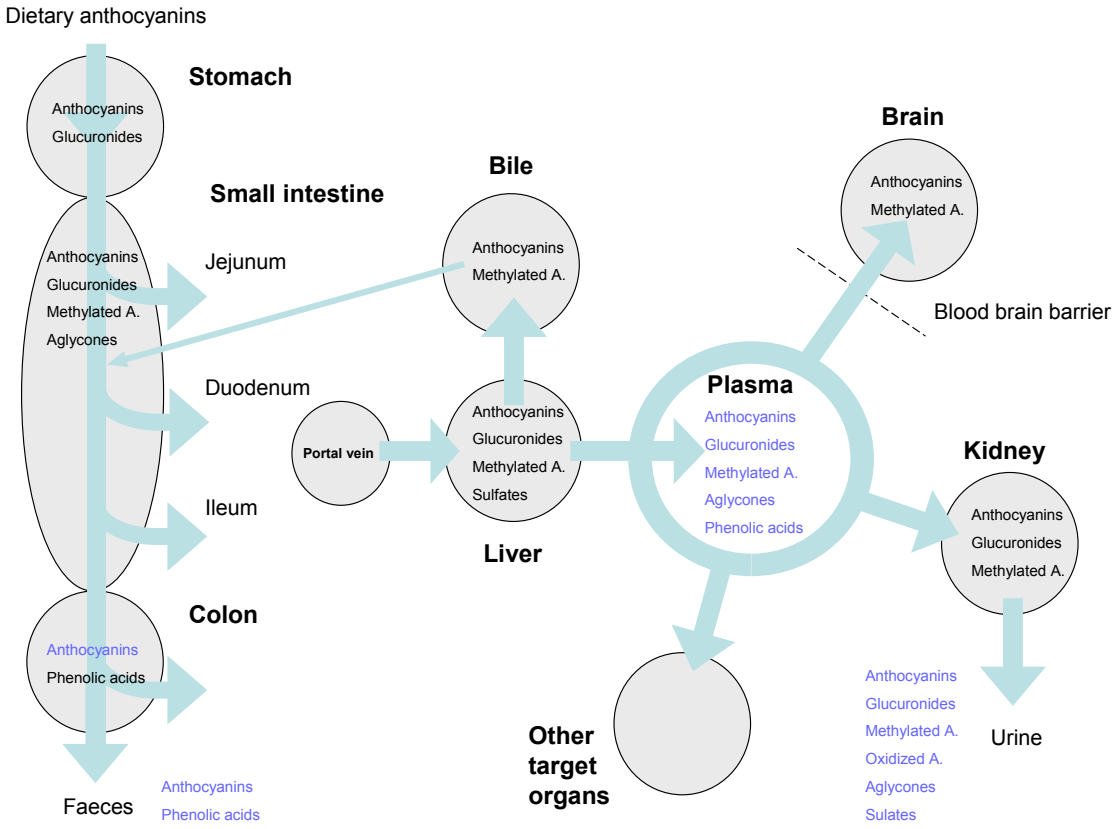

Figure 2-5 Absorption and bioavailability of anthocyanins in animals and humans. Blue letters denote evidence from human studies. A, anthocyanin.

Upon administration of black raspberry anthocyanins to rats by stomach intubation, total anthocyanins in the gastric lumen and tissue steadily decreased, whereas anthocyanin contents in the small intestinal lumen and tissue were highest at 120 min before decreasing. At 120 min, 7.5% of administered anthocyanins were found in small intestinal tissues, but anthocyanins were neither extensively delivered into the blood nor cumulatively retained [66].

Animal studies have revealed that anthocyanins are absorbed mainly in their intact glycosidic forms from the stomach and the small intestine [76-78]. Plasma levels of anthocyanins are governed by gastrointestinal uptake process, tissue distribution, degradation rate, and excretion to urine and bile, among other factors. Plasma concentrations peak between 15 and 30 min after oral uptake. With few exceptions, maximal plasma concentrations do not exceed nanomolar ranges and less than 1% of administered anthocyanins are found in urine, mainly as unmetabolized forms [70]. Moreover, anthocyanins, methylated forms and further metabolites were recovered from rat bile within 20 min, suggesting that liver metabolites are preferentially eliminated by bile [77]. Within 4 h post intravenous administration of anthocyanins, only 31% were recovered from urine and 13% from bile, which implies that considerable decomposition and tissue accumulation occurs [79]. Following 12 to 15 days of feeding

an anthocyanin enriched diet, anthocyanins distributed to rat stomach, jejunum, liver, kidney, heart, adipose, bladder, prostate, testes and brain tissues. In addition to native forms, methylated forms were identified in urine, plasma, jejunum, kidney, liver, adipose and bladder tissues, glucuronides were identified in urine, plasma, jejunum, liver, kidney, heart, prostate and testes tissues, and aglycones in jejunal tissues and plasma, suggesting the involvement of catechol-*O*-methyl-transferases, UDP-glucuronosyl transferases, and β-glucosidases [80,81]. Glucuronides were also found as pelargonidin main metabolites in rat plasma, urine, kidney, liver and lung, while *p*-hydroxybenzoic acid was identified as a phenolic metabolite in plasma and urine [82]. In rats receiving cyanidin-glc per stomach intubation, protocatechuic acid was detected in an eight-fold concentration in plasma compared to the native anthocyanin. Moreover, it was demonstrated that in rat plasma protocatechuic acid can only be efficiently generated from cyanidin and not from cyanidin-glc. Therefore, phenolic acids produced from anthocyanins or their aglycones in the intestine may be absorbed or aglycones may be degraded due to their instability in plasma [23]. In pigs receiving a blueberry supplemented diet for four weeks, anthocyanins were shown to accumulate in liver and eye tissues, and substances were not detectable in plasma or urine following fasting for 18-21 h. Anthocyanin accumulation was even shown for animals fed the basal diet, implying that low levels of anthocyanins may be highly bioavailable and well-retained in tissues [83]. Finally, absorption and metabolism of anthocyanins may be substantially determined by their aglycone structures and sugar moieties [84,85].

About 50 studies have been conducted on humans with respect to anthocyanin bioavailability, which are tabularized in the appendix. Inter-study comparisons, however, are complicated by differences in raw materials, administered doses, study conditions and lengths, and parameters of interests, e.g. the occurrence of metabolites and degradation products. As anthocyanin sources served blueberries, elderberries, chokeberries, boysenberries, lingonberries, blackberries, black raspberries, black currants, strawberries, cranberries, acai berries, red grapes, hibiscus, blood oranges, red cabbage, purple carrots, purple sweet potatoes and red onions, and among administration forms counted native fruits, juices, concentrates, powders, extracts and filled capsules. With few exceptions, single doses were administered, usually ranging from three-digit mg amounts to several grams of anthocyanins. It has consistently been observed that aglycone structures, sugar moieties, acylation patterns, concentrations and sources of individual compounds and coadministration of other food compounds impact the absorption, metabolism and excretion of anthocyanins. Moreover, great interindividual variations have been found.

Some indication for the fate of anthocyanins upon oral uptake is given by studies on ileostomy patients. Substances recovered from ileostomy fluids would reach the colon under physiological circumstances. Recovery rates of anthocyanins after the oral ingestion of lowbush blueberries ranged between 28 and 85% [86] and upon consumption of raspberries between 6 and 93% [87]. All anthocyanins passed the small intestine within 6-8 h after consumption and aglycones [86], sulfates or glucuronide metabolites were not identified [87]. Regarding sugar moieties, glucosides seem to be metabolized or absorbed most extensively, followed by galactosides (gal) and arabinosides (ara). In terms of the aglycones, cyanidin and delphinidin glycosides disappeared from the small intestine to a larger extent compared to petunidin and malvidin glycosides [86].

Maximal anthocyanin plasma concentrations are achieved between 0.5 and 4 h after ingestion, but primarily between 1 and 2 h, advocating a rapid absorption from the stomach. Peak plasma concentrations range from one-digit up to three-digit nanomolar values and anthocyanin clearance from circulation occurs readily with plasma half-lives of 1.3 to 7 h. Predominantly native glycosylated anthocyanins are detected in plasma. As exceptions, small amounts of methylated derivates were identified after administration of chokeberry extract [88,89], cyanidin aglycone was discovered upon elderberry extract treatment [90,91], and glucuronidated anthocyanins were found upon intake of chokeberry extract [88,89] and red wine extract [92]. After strawberry consumption, glucuronides even constituted the main anthocyanins recovered from plasma [93]. Upon blood orange consumption, 44% of ingested anthocyanins were found as protocatechuic acid in plasma [24]. With respect to phenolic metabolites, however, precursor molecules other than anthocyanins have to be considered as well. Although correlations between plasma bioavailability and structural features are inconclusive, nonacylated forms appear to become more efficiently absorbed than acylated anthocyanins [24,94-96].

It was also shown that cereal and rice cake can delay and cream and milk can additionally decrease plasma absorption [93,97-99], whereas phytic acid was able to enhance and increase anthocyanin absorption [100]. Moreover, it was demonstrated that with rising doses ingested, anthocyanin plasma bioavailability may become reduced as well as increased [95,98,101,102].

While the majority of studies describe that less than 0.1% of ingested anthocyanins are recovered from urine, 10 studies find values between 0.1 and 5%. Maximal urinary concentrations are detected between 0.5 and 12 h, and most frequently between 2 and 4 h. In contrast to plasma results, many studies detect the occurrence of different metabolites along with the native glycosylated forms. Anthocyanin glucuronides are

described in 13 studies, constituting up to 90% of excreted anthocyanins [103,104]. Moreover, methylated forms are reported in eight studies, five surveys find aglycone forms, three trials discover sulphated anthocyanins, and oxidized forms and loss of hydroxyl groups are described as well. This suggests that kidney may be a site of extensive anthocyanin metabolism. Distinct preferences for urinary excretion of certain anthocyanin chemical structures have not been observed, except of a higher elimination of nonacylated compared to acylated forms [95,105]. With respect to influence of other food ingredients, urinary elimination was delayed by cream [93], reduced and delayed by sucrose [106], increased by phytic acid [100], and no influence was described for rice cake [98]. Similar to plasma findings, contrary results have been described on the effect of the administered dose on the percent anthocyanin excretion, with higher anthocyanin doses giving rise to increased [98], unchanged [103,107] or decreased percent excretion values [95,101,105]. As concerns faecal elimination, along with 1.9 nmol/g native blood orange anthocyanins the 150-fold amount of protocatechuic acid was recovered, which may at least in part derive from anthocyanin precursors [24]. Thus, a majority of liver metabolites may be excreted with bile before entering systemic circulation.

2.2.1.3 Anthocyanin brain bioavailability

Brain bioavailability of anthocyanins is essential for *in vivo* neuroprotection and is determined to a large extent by the blood-brain barrier (BBB). The blood–brain barrier is formed by the endothelium of brain microvessels under the inductive influence of associated cells, especially astrocytes. Features that distinguish the brain endothelium from that of other organs include complex tight junctions, a low density of pinocytotic vesicles, and the expression of specific uptake and efflux transport systems and metabolic enzymes [108,109]. The blood-brain barrier ensures that neurotoxic metabolites are excluded from the brain, but allows essential nutrients to pass across, and presents a major hurdle that must be overcome for a potential therapeutic substance targeting a neurological condition [110].

Indications for anthocyanin brain bioavailability are given from studies on endothelial cell lines. Upon exposure times exceeding 6 h, cyanidin-rutinoside (rut) and pelargonidin-glc were taken up by mouse and rat brain endothelial cells. Moreover, both anthocyanins crossed an *in vitro* model of the BBB [111], suggesting transcellular permeability of anthocyanins.

In vitro evidence substantiates anthocyanin permeation through the blood-brain barrier. In mice, a single oral dose resulted in trace amounts of anthocyanins in brain tissue

[112]. Anthocyanidins may cross the blood-brain barrier as well, as pelargonidin aglycone was detected in brain after oral administration of pelargonidin on rats [82]. Anthocyanins were shown to reach the rat brain within 30 min after administration. Following a 15 day anthocyanin supplemented diet, native anthocyanins plus one methylated form were recovered in brain. Cyanidin-glc brain concentration (210 pmol/g) even exceeded plasma concentration (150 pmol/ml), suggesting anthocyanin accumulation in brain tissues [80]. A study on anesthetized rats corroborated these findings. Within few minutes, anthocyanins reached the brain and were detected at concentrations averaging 192 ng/g tissue. Malvidin-glc concentrations in brain, ranging from 0 to 236 ng/g tissue, were similar to plasma concentrations ranging from 23 to 380 ng/ml, and its brain p-coumarate ester concentrations of 0 to 357 ng/g even exceeded plasma concentrations of 0 to 34 ng/ml [113]. Correlation of plasma and brain levels were also reported in a study on rats applying a 3 months blueberry supplemented diet. In plasma, 480 pmol/ml anthocyanins were found, whereas hippocampus and cortex concentrations amounted to 450 and 460 pmol/g tissues, respectively [19]. Moreover, a variety of anthocyanins were detected in rat cerebellum, cortex and hippocampus after a 8-10 week blueberry-enriched diet, including glucosides, galactosides and arabinosides of cyanidin and malvidin, plus peonidin-ara and delphinidin-ara [114].

An accumulation of anthocyanins was also confirmed for pigs receiving a blueberry enriched diet for four weeks. Although no anthocyanins were found in plasma and urine after pigs were fasted for 18-21 h, they were identified in cortex and cerebellum at 0.88 and 0.66 pmol/g tissue [83]. Similar anthocyanin concentrations were detected in pig cortex, cerebellum, midbrain and diencephalon after an eight week blueberry supplementation and 18-21 h fasting, suggesting a longer residence time of anthocyanins in brain tissues compared to plasma.

As structural features of anthocyanins may be major determinants for compounds' stability, metabolism and brain uptake, they may finally affect anthocyanin brain bioavailability. Malvidin-glc and malvidin-gal were the predominant anthocyanins found in pig brains, which may be due to preferential brain uptake, relative stability or additional generation from delphinidin and petunidin glycosides catalyzed by liver or brain catechol-*O*-methyltransferases. Furthermore, glucuronides were identified in all brain regions investigated, reaching approximately one tenth of the levels observed for their parent glycosides, with delphinidin glucuronide being the most prevalent conjugate. Surprisingly, various anthocyanins and glucuronide metabolites were detected in all four brain regions in pigs receiving the control diet, being supportive of a high brain bioavailability of anthocyanin traces [115].

2.2.2 Proanthocyanidins

2.2.2.1 Proanthocyanidin stability

In terms of proanthocyanidin gastrointestinal stability, inconclusive results derive from *in vitro* and *in vivo* studies. Incubation of cocoa PAs (tetramers to hexamers) with simulated gastric juice *in vitro* showed that PAs are hydrolyzed to mixtures of epicatechin monomers and dimers [116]. Further *in vitro* fermentation of purified B-type proanthocyanidin dimers with human microflora yielded phenolic acids as main metabolites. Among those count 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid (3-HPA), 4-hydroxyphenylacetic acid (4-HPA), 3-(3-hydroxyphenyl)propionic acid (3-HPP), plus various hydroxylated phenylvaleric acids and phenylvalerolactones, whereas flavan-3-ol monomers (+)-catechin and (-)-epicatechin were not formed by microbial degradation [117]. Similarly, *in vitro* degradation of PAs with DPs of 4 and higher by human colonic microflora generated 4-HPA, 3-HPA, 3-phenylpropionic acid, 3-HPP, 3-(4-hydroxyphenyl)propionic acid (4-HPP) and 5-(3-hydroxyphenyl)valeric acid (3-HPV) [118].

In vivo, upon oral administration of grape seed proanthocyanidin extract (GSPE) to rats, no indication for PA depolymerization was given [119]. In accordance, proanthocyanidins up to pentamers from cocoa beverage were demonstrated to be stable during stomach transit in humans. This implies that cocoa PAs reach the intestine intact and may be degraded to metabolites by the intestinal microflora [120].

2.2.2.2 Proanthocyanidin bioavailability and metabolism

Bioavailability is a major requirement for native proanthocyanidins or their metabolites to exert physiological effects on humans. Since PAs in general are very high molecular weight molecules, it is unlikely that they are absorbed as intact native forms [48].

In vitro absorption of PAs was investigated with a model of the absorptive intestinal epithelium. While PA dimers and trimers had similar permeability coefficients, permeability of PAs with a DP of 6 was about 10 times lower [121].

In vivo, PA dimers and trimers were found in rat plasma after administration of GSPE [122], as were orally administered proanthocyanidins B2 and B3 [123]. A further study detected PA dimers, trimers, tetramers and pentamers in rat plasma, peaking at 2 h. Interestingly, polymeric PAs were shown to favor absorption of PA oligomers without being absorbed themselves [124]. Moreover, *ex vivo* perfusion of rat jejunum with B2 and B5 epicatechin dimers led to high levels of unmetabolized and unconjugated

epicatechin on the serosal side of enterocytes, suggesting an energy-dependent cleavage during transfer [125].

PA dimers and trimers were also identified in rat urine after GSPE consumption [119], as were proanthocyanidin B2, epicatechin and 3'-*O*-methyl-epicatechin in plasma and urine after administration of proanthocyanidin B2 [126]. Further it was demonstrated that the degree of polymerization exerts a major impact on PA degradation in rats, with higher pds leading to less metabolites identified in urine. Among main metabolites counted vanillic acid, p-hydroxybenzoic acid, 3-HPP and 3-hydroxycinnamic acid (3-HC) with respect to proanthocyanidin B3, and 3-HPP, 3-HPV and 3-HC with respect to C2 and higher polymers [127].

Contrary results were obtained from studies finding neither parent PAs, nor conjugates or monomers in rat plasma or urine upon administration of a diet supplemented with dimeric and trimeric PAs or GSPE [127,128]. These findings suggest that PAs are not hydrolyzed into monomers in the rat stomach or intestine. In line with these results, only trace amounts of PA metabolites were detected in rat colon upon GSPE feeding, and PAs were excreted in faeces without any indications for depolymerization [119].

In humans, only dimeric PAs have been detected in plasma. Proanthocyanidin B2 was identified in human plasma 0.5 h after cocoa consumption, yielding peak concentrations of 41 nM and 100 nM after 2 h [129,130], and B1 was detected in human serum 2 h after GSPE intake at 11 nM [131]. Potential metabolites cannot conclusively be assigned to proanthocyanidin precursors, since only complex flavonoid mixtures have been administered and phenolic acids identified may also derive from other polyphenols [132,133].

2.2.2.3 Proanthocyanidin brain bioavailability

In terms of proanthocyanidin brain bioavailability, available data are scarce. While passive diffusion of substances through the BBB is unlikely due to their size, crossing mediated by active transporters has not been investigated to date. Upon oral administration of epicatechin to rats, a study reported the presence of the epicatechin glucuronide and 3'-*O*-methyl epicatechin glucuronide in the rat brain tissue [134], suggesting that proanthocyanidin metabolites found in plasma may be further candidates to pass the blood-brain barrier.

2.2.3 Transport theories

Anthocyanins are unique compared with other flavonoids in that they are absorbed intact as glycosides, although the mechanism of absorption is not known. Since passive diffusion is unlikely due to their molecule size and charge, carrier mediated transcellular transport may be hypothesized [135].

Bilitranslocase, an organic anion membrane carrier, is proposed to be involved in anthocyanin absorption, since uptake of malvidin-glc into human endothelial cell monolayers was inhibited with impaired bilitranslocase function [136]. Moreover, anthocyanins appeared as competitive inhibitors of bilitranslocase transport activity in membrane vesicles. Therefore, it is hypothesized that at the gastric level interaction with bilitranslocase may promote anthocyanin transport from the intestinal lumen into the epithelial layers of the gastric mucosa, favoring their transfer to the portal blood, and at the hepatic level from the portal blood into liver cells [137]. Since bilitranslocase activity was also competitively inhibited by malvidin-glc in kidney membrane vesicles, anthocyanin uptake from blood into kidney tubular cells is likely to be mediated by kidney bilitranslocase as well [138]. A bilirubin-binding motif identified in bilitranslocase has also been found in the central nervous system (CNS), advocating that anthocyanins may enter the brain by means of a carrier similar to bilitranslocase [80,139]. This hypothesis is also supported by the fact that anthocyanin tautomers of anionic quinoidal bases at physiological pH values meet structural requirements for bilitranslocase substrates [137].

Further evidence for possible mechanisms of absorption is given by a study applying a Caco-2 monolayer as a model of the absorptive intestinal epithelium. Enhancement of anthocyanin transport upon preincubation with anthocyanins raises the hypothesis that anthocyanins may interfere with the transporters responsible for their own transport. While sodium-dependent glucose cotransporter 1 (SGLT1) and facilitative glucose transporter 5 (GLUT5) expression did not change, anthocyanins increased facilitative glucose transporter 2 (GLUT2) expression by 60%. Moreover, anthocyanins were demonstrated to decrease glucose uptake, in contrast to an aglycone, suggesting a competition between glucose and anthocyanins for GLUT2 transport rather than glucose cotransport [72]. These *in vitro* findings are in line with results of a human bioavailability study, showing that sucrose can lead to a reduced and delayed excretion of anthocyanins upon elderberry juice consumption [106]. Nevertheless, transport of anthocyanins across Caco-2 monolayers was not efficient [72,73] or not observed at all [74]. Possible explanations derive from quercetin-glc transport studies across Caco-2 monolayers. Quercetin-glc was absorbed across the apical membrane, but accumulated within cells without further translocation to the basolateral side [140].

Subsequently, it was demonstrated that apical multidrug resistance-associated protein 2 (MRP2) mediated quercetin-glc efflux, thus limiting absorption across the monolayer [141].

Accordingly, studies addressing possible mechanisms of anthocyanin transport indicate that permeability of the intestinal barrier and BBB for anthocyanins may also be affected by efflux transporters expressed at the endothelial surfaces. Efflux transporters of the ATP-binding cassette (ABC) gene family are major determinants of drug distribution to, and elimination from the CNS, of intestinal absorption, and of hepatobiliary excretion [142,143]. Localized in the apical membranes of BBB endothelial cells, enterocytes and hepatocytes [144,145] (figure 2-6), they mediate the active extrusion of nutrients, toxins, drugs and many metabolites back into the capillary lumen and intestine [146-150]. Among ABC efflux transporters count the multidrug resistance protein 1 (MDR1, P-glycoprotein) and the breast cancer resistance protein (BCRP). Both are prominently expressed in organs important for absorption (small intestine), distribution (placenta and BBB) and elimination (liver, kidney, small intestine) of drugs [151,152], and are also found in several tumors [153,154].

Therefore, further investigations are demanded to establish possible roles of ABC efflux transporters in poor anthocyanin absorption and anthocyanin brain bioavailability.

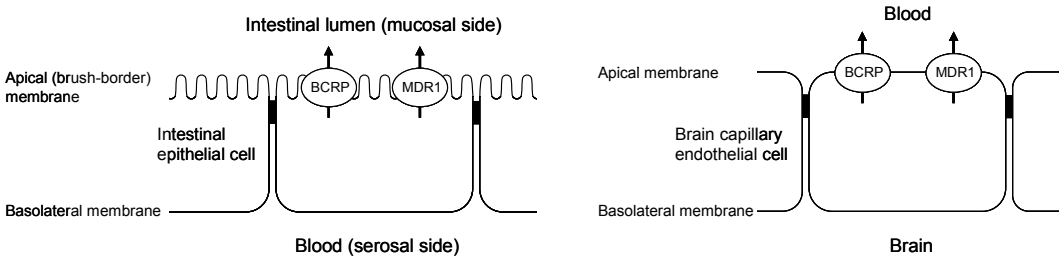

Figure 2-6 Localization of ABC transporters MDR1 and BCRP in intestinal epithelial cells and brain capillary endothelial cells [144,145,147,148,152].

More recent research has demonstrated that in addition to phase I and II metabolism, transport mechanisms are critical lines in xenobiotic defense [155]. Thus, ABC transporters not only limit the oral availability of xenobiotics, but may cause food-drug interactions depending on the presence of inducers or inhibitors of drug transport. Specific information on anthocyanins' potential to interact with ABC transporters should therefore help to gauge the likelihood of adverse effects from dietary intake of berry products and related nutritional supplements [156-158].

2.3 Health benefits of anthocyanins and proanthocyanidins

2.3.1 General health benefits

2.3.1.1 Clinical and preclinical observations

Beyond the neuroprotective effects that have been ascribed to anthocyanins and proanthocyanidins, berry constituents may possess additional functionalities, e.g. in preventing cardiovascular disease. Prospective studies have reported statistically significant inverse associations between flavonoid intake and the incidence of cardiovascular disease or mortality [159-161]. With respect to anthocyanins, a blueberry-enriched diet effectively prevented myocardial infarction in rats [162]. In atherosclerotic patients with carotid artery stenosis or coronary artery disease, pomegranate juice and red grape juice supplementation have helped reduce endothelial dysfunction, systolic blood pressure and stress-induced ischemia [163-166]. In a prospective study on more than 34,000 postmenopausal women, significant inverse associations between anthocyanin intake and coronary heart disease, cardiovascular disease and total mortality have emerged [167]. Proanthocyanidins' cardioprotective effects have been addressed and confirmed in a clinical trial of high proanthocyanidin dark chocolate intake [168]. Taken together, there is increasing evidence of berry constituents' potential to promote cerebrovasular circulation and cardiovascular health.

Finally, animal studies have argued in favor of anthocyanins' and proanthocyanidins' anticancer properties following dietary supplementation [169-171]. While a pilot clinical trial of anthocyanin-rich bilberry extract slowed tumor proliferation, with anthocyanins found in tumor tissues at 180 ng/g [172], epidemiological studies have, however, failed to ascertain such effects in larger populations [173,174].

2.3.1.2 Cellular functionalities

General health benefits associated with diets rich in anthocyanins' and proanthocyanidins' may be ascribed to multi-level biological activities. A majority of protective effects have been related to berry substituents' antioxidant properties [102,175-191].

Since oxidative damages have been discussed as precursors to heart disease, hypertension and atherosclerosis [192], anthocyanins may exert beneficial effects by

reducing lipid and protein oxidation [16], increasing levels of antioxidant enzymes superoxide dismutase and glutathione peroxidase in blood [192], and increasing blood glutathione levels and glutathione reductase activity [193,194]. In clinical trials, pomegranate juice consumption decreased susceptibility to plasma lipid peroxidation and low density lipoprotein (LDL) aggregation [195], and red grape juice reduced plasma oxidized LDL [196]. In blood of haemodialysis patients, red fruit juice decreased malondialdehyde (MDA) and protein carbonyls, markers of lipid and protein peroxidation, respectively [193]. As GSPE mitigated oxidative damage in a rodent atherosclerosis model [197], proanthocyanidins may also promote human cardiovascular health by reducing LDL oxidation [198].

Beside exerting antioxidative activities, anthocyanins are suggested to mediate protection from atherosclerosis and cardiovascular disease by inhibition of angiotensin converting enzyme [199-201], reduction of plasma total cholesterol and elevation of high density lipoprotein (HDL) cholesterol [196,202,203], prevention of platelet aggregation [195,204,205], and elevation of serum activity of LDL oxidation counteracting paraoxonase-1 [163]. Finally, proanthocyanidins may provide protection from cardiovascular disease by downreglulation of proapoptotic genes, reduction of cardiomyocyte apoptosis [190,191], modulation of total cholesterol and triglyceride levels [197] and prevention of platelet aggregation [206].

As oxidative damage has also been considered as a precursor for cancer [192], berry constituents may exert anticancer effects by attenuating oxidative chromosomal damage [193,207-209]. Protection from cadmium chloride-induced nephrotoxicity by GSPE further suggests that berrry constituents defend target organs from carcinogenic agents by means of an antioxidative mechanism [210]. Moreover, an antioxidative mechanism of photoprotection by GSPE was revealed [171].

Alongside employing antioxidative mechanisms, anthocyanins may exert anticancer effects [211-214] by inhibition of angiogenesis [176], induction of apoptosis [215], blocking of different cell cycle phases and affecting regulator proteins [216,217]. Among these, inhibition of various receptor tyrosine kinases [218] and prevention of mitogen-activated protein kinase (MAPK) pathway activation have been discussed [217,219].

Further mechanisms by which proanthocyanidins may mediate antiproliferative activities may involve arrest of cell cycle in G_1 and G_2 phases [220,221], downregulation of antiapoptotic and upregulation of proapoptotic effectors [222]. Finally, inhibition of epidermal growth factor receptor (EGFR) [223], MAPK protein

activation, nuclear factor κB (NF-κB) activation and expression of its target genes have been suggested [224], as well as inhibition of topoisomerases I and II [223].

2.3.2 Neuroprotection

2.3.2.1 Neuroprotective effects

2.3.2.1.1 Target pathologies

Neurodegenerative disease is defined by the progressive loss of neurons leading to functional impairment as in Alzheimer's disease (AD), Parkinson's disease, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), among others [225]. *In vitro* and *in vivo* models of neurodegenerative disease can help assess neuroprotection that is conferred by candidate xenobiotics.

AD and PD are the most prevalent neurodegenerative diseases and warrant intensive research on effective strategies to counteract these age-related disorders. AD manifests clinically as a progressive impairment in episodic memory and intellectual functioning, affecting language, perceptual, and perceptuomotor skills [226]. Among the primary cardinal lesions associated with Alzheimer's disease count neurofibrillary tangles and senile plaques. Neurofibrillary tangles consist of accumulations of abnormally phosphorylated tau proteins in certain neurons, while senile plaques consist of a central core of β-amyloid (Aβ), surrounded by abnormally configured neuronal processes or neurites. This is accompanied by the loss of synaptic components [227].

PD presents with a classical triad of motor symptoms: akinesia, rigidity and tremor. Many patients also suffer from non-motor symptoms, including disturbances of autonomic and cognitive functions [228]. The progressive degeneration of midbrain dopaminergic neurons in the substantia nigra pars compacta is a cardinal feature of Parkinson's disease pathology [229]. Intraneuronal inclusions and Lewy neurites are the defining neuropathological characteristics and hallmark of PD, with α-synuclein forming the major filamentous component [230].

2.3.2.1.2 Neuroprotection mediated by anthocyanins and proanthocyanidins

Various anthocyanin and proanthocyanidin neuroprotective effects on cellular and tissue levels have been described. While cyanidin-glc exerted cytoprotective effects in neuronal cells [7], blueberry constituents reduced neuronal loss induced by excitotoxic

kainic acid [13] and GSPE partially protected the neuronal tissue upon *O*-ethyl-S,S-dipropyl phosphorodithioate (MOCAP) induced neurotoxicity in rodents [210]. Regarding Alzheimer's disease, cyanidin-glc was demonstrated to inhibit Aβ-induced neuronal viability loss *in vitro* [231].

Several efforts have been made to find nutritional strategies based on berry fruit constituents to combat age-related deficits observed in neurodegenerative disorders. A growing number of behavioral investigations report neuroprotective effects of berry constituent. In rats, blueberries have provided protection against age-related decrements in cognitive and learning performance [13,14,232] and have enhanced short-term and working memory [17]. Moreover, in aged rats, blackberries and red grape juice have improved cognitive function [9,18]. While blueberry constituents have benefited object recognition and spatial working memory [19,233], plum juice has equally exerted positive effects on memory [234]. In a further study, mulberry extract improved learning and memory in senescence-accelerated mice [235], confirming earlier findings on blueberry and strawberry supplementation with regard to spatial learning and memory [236]. Beneficial effects on long-term memory are indicated by behavioral studies on blueberry supplemented mice [237]. Similarly, GSPE rich in proanthocyanidins has exhibited memory enhancing effects [238].

In humans, daily supplementation with blueberry juice for 12 weeks resulted in improved memory performance of subjects with early memory decline [6]. In contrast, effects of six weeks' supplementation with cranberry juice on neurocognitive performance did not reach significance. These negative results may, however, be due to study duration or the superior baseline performance of a healthy elderly population [239].

Finally, nutritional epidemiology data confine to behavioral effects induced by mixtures of flavonoids. Studies indicate that inclusion of antioxidant-rich foods in the diet can improve cognitive functioning in humans [240]. Thus, in elderly non-demented subjects, elevated dietary intake of flavonoid-rich foods was associated with better cognitive function and attenuation of cognitive decline over a period of 10 years [241]. In aging women, vegetable consumption was inversely associated with cognitive decline [242]. Other studies have revealed that attention to daily fruit and vegetable consumption and adherence to a "Mediterranean diet", mainly based on vegetables, fruits, beans and nuts, can effectively decrease the risk of developing dementia in aging humans [243].

In summary, these results suggest that berry supplementation has considerable potential to retard or even prevent the age-related decline in cognitive function.

Additional central nervous functionalities include improvements of motor performance in rodents following blueberry [14,237], blackberry and red grape juice supplementation [9,18].

Oral administration of blueberry constituents has also been associated with protection from ischemia-induced damage in rats [244-246]. A further study on mice showed that mulberry fruit extract and cyanidin-glc decreased infarct volume, corroborating that anthocyanins are the active principles of berry fruits [7]. This is backed by a meta-analysis of prospective observational studies showing that risk of stroke decreased by 11% for each additional portion of fruit per day [247].

Neuroprotection by berry constituents in animals is also illustrated by the prevention of N-methyl-D-aspartic acid (NMDA)-induced retinal damage [15] and the enhancement of hippocampal plasticity [248]. Moreover, reversal of age-related changes in temporal processing speed in the rat primary auditory cortex was found upon dietary supplementation with berry constituents, which may forestall age-related hearing loss [249].

Preliminary data point at antianxiety effects of blueberry supplementation in mice [237] and at antidepressive effects in humans [6]. Furthermore, proanthocyanidins may account for antinociceptive effects [250].

It would appear that berry constituents hold promise in ameliorating sensory perception although the evidence remains conflicting for certain modalities, e.g. night vision [251].

Neuroprotection by berry constituents is rationalized by anthocyanin brain bioavailability (see chapter 2.2.1.3). For instance, demonstration of anthocyanins' uptake into rat cerebellum, cortex and hippocampus following dietary supplementation with blueberries [114] further corroborates anthocyanins' potential to improve cognitive performance including learning and memory. Despite the spectrum of neuroprotective effects elicited by anthocyanin-rich berry fruits in animals, further clinical research is warranted to firmly establish the utility of anthocyanins and other berry constituents in the prevention of chronic central nervous disease.

2.3.2.2 Neuroprotective mechanisms

2.3.2.2.1 Pathomechanisms of neurodegenerative diseases and aging

In sporadic forms of neurodegenerative diseases common findings include alterations in the homeostasis of antioxidants and oxidation, elevations of iron and nitric oxide

levels, inflammation, activation of apoptosis pathways and glutamatergic toxicity [252]. Usually, these processes do not appear independently, but are rather closely interrelated. Aging is considered a major risk factor for neurodegenerative disease [253], as are recurrent or chronic systemic infections [254], and environmental agents such as heavy metals, pesticides and organic solvents [255].

The imbalance between cellular production of reactive oxygen species (ROS) and the ability of cells to defend themselves against ROS is referred to as oxidative stress [256]. Of all explanatory factors, oxidative stress-related processes likely represent the most widely accepted common feature of neurodegenerative diseases and aging. The brain – an organ that requires high metal ion concentrations to maintain many of its functions – is particularly vulnerable to oxidative stress, and demonstrates only little regenerative capacity [110]. ROS may oxidize cell membrane lipids, DNA, proteins and enzymes involved in neuronal survival and communication, causing cellular damage and death [8,253]. Many neurodegenerative disorders are associated with the accumulation of abnormal protein assemblies that can act as triggers of cellular stress and neuroinflammation [257]. Other consequences of oxidative stress comprise the disruption of calcium homeostasis, alterations in cellular signalling cascades, and changes in gene expression. As a result, neurotransmitter receptors and other structures may become even more vulnerable to oxidative stress [258]. The long-term effects of oxidative stress and inflammatory insults are equally seen in normal aging, but would appear to be more extensive in the progression of neurodegenerative disease [236].

Brain iron has also been implicated in neurodegeneration. Both elevated tissue iron and miscompartmentalization of copper and zinc have been observed in neurodegenerative diseases [259], with an age-related increase in iron staining and ferritin immunoreactivity primarily in microglia and astrocytes of the hippocampus, cortex, cerebellum, basal ganglia and amygdala [260]. Neurons in the aged brain exhibit alterations in the ability to regulate intracellular iron concentrations, leading to deficits in neuronal communication and synaptic plasticity [8]. Overall, iron localization coincides with the production of ROS, which may place these areas of the brain at increased risk of neurodegeneration [260,261]. As ROS were found to induce the heme-degrading enzyme heme oxygenase 1 (HO-1) in rat astrocytes, the subsequent liberation of free cytosolic Fe^{2+} [262] may therefore exacerbate brain aging processes by engaging in a neuropathological vicious cycle of ROS generation [263].

Free iron is a potential source of oxidative stress in that it catalyzes the conversion of hydrogen peroxide into highly reactive hydroxyl radicals by the Fenton reaction. In

addition, iron-dependent lipid peroxidation may generate potentially toxic peroxyl and alkoxyl radicals [264], and free ferrous iron may convert neutral catechols (e.g. dopamine) to neurotoxic *o*-semiquinone intermediates [265]. Furthermore, aberrant redox metal interactions with key proteins promote protein aggregations, oxidative stress and neuronal death [110].

Besides oxidative stress-linked processes, CNS inflammatory events may play a major role in functional impairment related to aging [258] and chronic CNS disease [257,266]. Inflammation can be triggered by infectious agents, injurious chemicals or physical insults. It can also be initiated from within the organism, e.g. by diseases affecting the immune system or the nervous system [257,266]. Conditions such as ischemic brain injury, traumatic brain and spinal cord injuries, epileptic seizures, AD, PD, HD, multiple sclerosis and ALS all feature an inflammatory component [267,268].
The term “inflammation” refers to complex series of active defense reactions executed by a host against diverse insults designed to remove or inactivate noxious agents and to inhibit and reverse their detrimental effects such as tissue damage. In neurodegenerative disorders, this reaction may be induced by protein aggregates and other abnormally modified cellular constituents that are byproducts of oxidative stress. Other triggers include molecules released from or associated with injured neurons or synapses, and disregulation of inflammatory control mechanisms [257]. In aging and neurodegenerative disease, neuronal deficits arise with concomitant activation of glia cells [8,257] and the production of cytokines, growth factors, and complement proteins [258].

Excitatory neurotransmission has emerged as a further mechanism of neuronal damage, causing excitotoxic necrosis of cortical and subcortical neurons, a phenomenon commonly known as excitotoxicity [226].
However, despite significant progress in the field, the primary events in neurodegeneration and age-related decline remain to be determined [252]. In order to develop strategies for prevention and, ultimately, for the treatment of these conditions, there is a pressing need to identify protective agents and to understand their mode of action.

2.3.2.2.2 Neuroprotection mediated by anthocyanins and proanthocyanidins

2.3.2.2.2.1 Antioxidative mechanisms

The contribution made by berry constituents' antioxidative properties to neuroprotection is substantiated by many *in vitro* and *in vivo* studies. *In vitro*, purple sweet potato extract attenuated ROS generation, lipid peroxidation and DNA fragmentation [269], while anthocyanin-rich fractions from boysenberry and black currant protected against H_2O_2-induced oxidative stress and cytotoxicity [270]. Moreover, anthocyanins inhibited oxidation of the neurotoxic dopamine metabolite 6-hydroxydopamine *in vitro* [271], and anthocyanins and potential metabolites counteracted cytosolic and membrane ROS formation [272]. *In vitro* evidence suggests that iron chelation may also prevent oxidative death [273]. As for proanthocyanidins, an antioxidant activity of GSPE has been demonstrated in neuronal cell cultures [188].

In rodents, bilberry extract led to a decline in parameters of lipid and protein oxidation in cortex, midbrain and cerebellum, and suppressed stress-induced changes of dopamine levels [16]. In senescence-accelerated mice, mulberry extract augmented antioxidant enzyme activity and diminished lipid peroxidation in brain [235]. It has been proposed that berry fruits' neuroprotective potential may partly be ascribed to enhanced expression of antioxidant heat shock proteins [274] and of other antioxidant enzymes [235]. Pretreatment of mice with GSPE conferred protection against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA damage in brain tissue [207]. Moreover, processed lychee extract rich in PA monomers, dimers and trimers reduced lipid peroxidation in mouse brain [275]. These results provide further indication of antioxidant mechanisms in proanthocyanidin neuroprotection.

Antioxidative effects may also be achieved by lowering brain iron levels as demonstrated with the orally administrated, blood-brain barrier-permeable chelator (R)-α-lipoic acid in rodents [276]. Chelators of iron have been found to prevent injury in animal models of neurological conditions and in a small pilot clinical trial in Alzheimer's disease [273]. Thus anthocyanins' and proanthocyanidins' chelating properties [277,278] warrant further research as putative mediators of antioxidant defense.

2.3.2.2.2.2 Antiinflammatory mechanisms

It has long been hypothesized that berry constituents' antiinflammatory properties play an essential role in counteracting inflammation-induced damage associated with neurological disorders and aging [10,16,235].

These properties are illustrated *in vitro*, by anthocyanins' inhibition of human neutrophil granulocyte 5-lipoxygenase (5-LOX) [279], anthocyanidins' suppression of calcium-insensitive nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression [280], and anthocyanins' and anthocyanidins' inhibition of COX-1 and COX-2 activities [281,282]. Moreover, anthocyanins reduced the release of inflammatory mediators such as interleukin-8 (IL-8), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) in human microvascular endothelial cells [283]. Grape seed proanthocyanidins displayed inhibitory effects on expression of vascular cell adhesion molecule (VCAM-1), which contributes to the cell adhesion process important in inflammation [284].

Limited data are currently available on berry constituents' antiinflammatory properties *in vivo* with regard to the CNS. One study suggests that anthocyanins may mediate neuroprotection by enhanced expression of antiinflammatory heat shock proteins in rats [274]. Assuming that anthocyanins' and proanthocyanidins' effects on inflammation in blood and non-neuronal tissues extend to the brain, a range of target effectors can be identified. Inhibitory effects on COX-2 activity were elicited upon dietary supplementation with sour cherry juice in rodents [192], while blueberry powder supplementation attenuated upregulation of tumor necrosis factor-α (TNF-α), IL-6, IL-10, iNOS, and MCP-1 in adipose tissue [285]. In a rat model of acute lung inflammation, blackberry anthocyanins led to a decrease in all test parameters of inflammation [286]. Orally administered cyanidin-glc, cyanidin, and protocatechuic acid successfully inhibited histamine-induced scratching behavior in mice [65]. GSPE proanthocyanidins, in turn, exhibited favorable effects on collagen-induced arthritis, an animal model of rheumatoid arthritis. Antiarthritic activities were associated with a reduction in the production of type-II-collagen specific immunoglobulin G2a (IgG2a) and inflammatory cytokines, plus the suppression of osteoclastogenesis [287].

In humans, consumption of red grape juice and grape peel anthocyanin extract lowered circulating MCP-1 levels [92,182], while dealcoholized red wine reduced MCP-1 stimulated monocyte migration *ex vivo* [288]. Dietary supplementation with bilberry and blackcurrant anthocyanins decreased levels of circulating proinflammatory chemokines, cytokines and inflammatory mediators such as IL-8, IL-4, IL-13 and interferon-α (IFN-α) [266]. Finally, red grape juice suppressed the release of platelet-dependent superoxide and soluble CD40L, a marker of platelet activation [289]. Proanthocyanidins'

antiinflammatory effects are corroborated by the outcome of a clinical trial involving systemic sclerosis patients supplemented with GSPE. In these subjects, the expression of adhesion molecules in plasma was attenuated and oxidative stress was reduced [290].

2.3.2.2.2.3 Further candidate mechanisms

Many additional cellular mechanisms are being discussed, by which berry constituents may exert neuroprotective activities.

Among these counts the direct impact on neuronal cell signalling [19,258,291] that may cause reductions in NF-κB expression, stress and apoptosis signalling [233]. These observations are complemented by attenuated caspase-3 activation and intracellular calcium increase from exposure to purple sweet potato extract [269]. Delphinidin effects on signalling are also shown by its ability to counteract the elevation of intracellular calcium levels and tau protein phosphorylation [292], and cyanidin and protocatechuic acid reduced apoptotic events including mitochondrial functioning loss and DNA fragmentation [272].

As berry constituents were shown to prevent or reverse declines in cerebellar noradrenergic receptor function [293] and blueberry and strawberry supplementation reduced changes in striatal dopamine release induced by radiation [236], anthocyanin effects on CNS signal transduction may also involve interactions with muscarinic cholinergic and striatal dopamine systems, receptor sensitivities [14,16,236,294] and cerebellar noradrenergic receptor function [293,295].

Brain acetylcholinesterase (ACE) may present a further target of neuroprotective berry polyphenols. Agonists at cholinergic receptors and inhibitors of acetylcholinesterase have long been used in the prevention and treatment of cognitive deficiencies and recent evidence suggests that blueberry extract may also act as an ACE inhibitor [296].

Neuroprotection by proanthocyanidins and GSPE may involve the expression of heat shock, neurofilament, and cytoskeletal proteins plus proteins involved in energy generation. The direction of PA-induced changes is contrary to changes observed in diseased brain and suggests that ingestion of GSPE is neuroprotective [297,298].

Inhibition of β-amyloid spontaneous aggregation by cyanidin-glc *in vitro* [231] and decrease of Aβ protein levels by mulberry extract in senescence-accelerated mice [235] point to further neuroprotective mechanisms of anthocyanins in terms of AD.

Finally, proanthocyanidins may influence the permeability of the blood-brain barrier as indicated by a study on rats administrating proanthocyanidin oligomers. They were demonstrated to prevent the permeability increase of brain microvessels induced by

collagenase injection, presumably by increasing the resistance of constituents of the capillary baseline lamina to proteolytic degradation [299].

2.3.2.2.2.4 Monoamine oxidases inhibition

The role of monoamine oxidases in neuroprotection is firmly established and monoamine oxidase inhibitors count among agents with a long tradition in the treatment of PD [300]. Monoamine oxidases are flavin-containing enzymes embedded in the outer mitochondrial membranes of neuronal and non-neuronal cells [301,302]. They catalyze the oxidative deamination of amines from both endogenous and exogenous sources, and thereby regulate concentrations of neurotransmitter amines, neurotoxins, xenobiotics and amines from food sources [303-305].

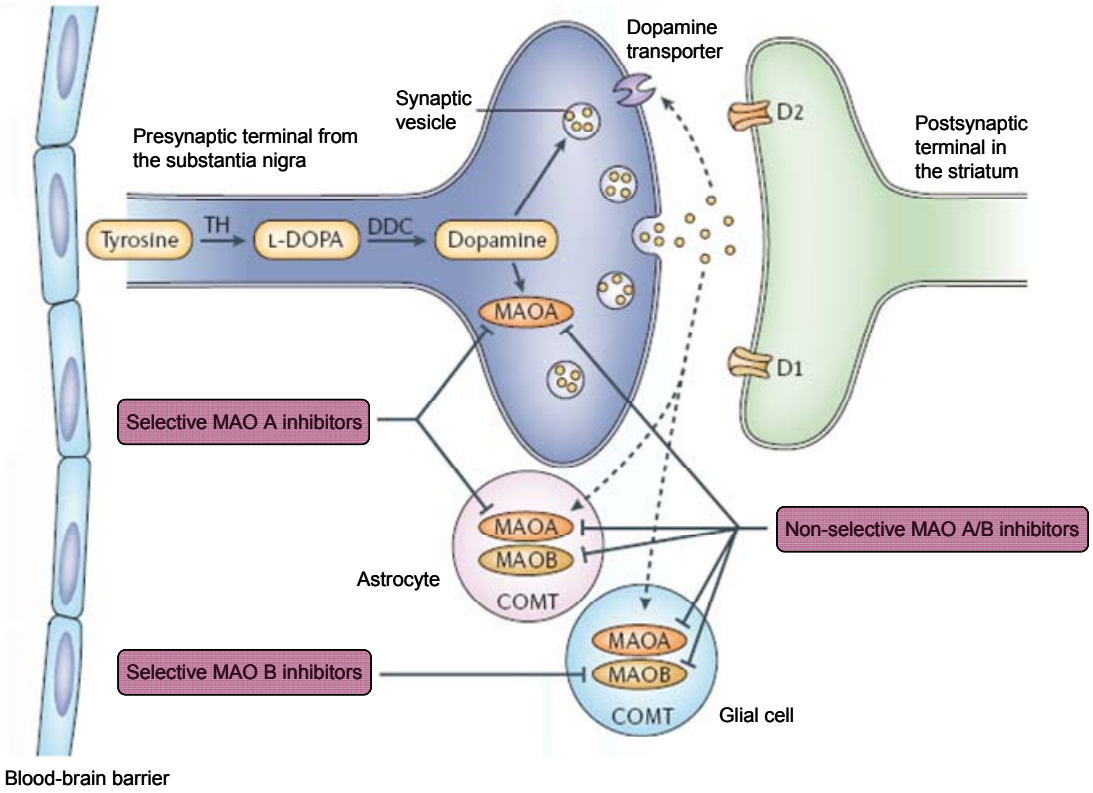

Figure 2-7 Pathways of dopamine synthesis in dopaminergic neurons and metabolism by MAO A and B in the human brain. Tyrosine passes the blood–brain barrier, is hydroxylated by tyrosine hydroxylase (TH) to L-3,4-dihydroxyphenylalanine (L-DOPA) and then decarboxylated by DOPA decarboxylase (DDC) to dopamine (DA) within the neuron. DA is metabolized by intraneuronal monoamine oxidase A (MAO A), and by glial and astrocyte MAO A and MAO B. Selective inhibitors of MAO A and MAO B do not alter the steady-state striatal dopamine levels, although chronic treatment with these drugs does enhance dopamine release. However, non-selective MAO A/B inhibitors increase the levels of dopamine in the striatum and other regions. D1, D2, dopamine receptors; COMT, catechol-*O*-methyltransferase. Figure adapted by permission from Macmillan Publisher Ltd: Nature Reviews Neuroscience [306], copyright (2006).

End products of these enzymatic reactions, aldehydes and hydrogen peroxide, play key roles in oxidative cellular injury [303,305]. Therefore, MAO inhibitors may mediate neuroprotection by modulating neurotransmitter concentrations in particular neurodegenerative conditions and by preventing oxidative events induced by hydrogen peroxide.

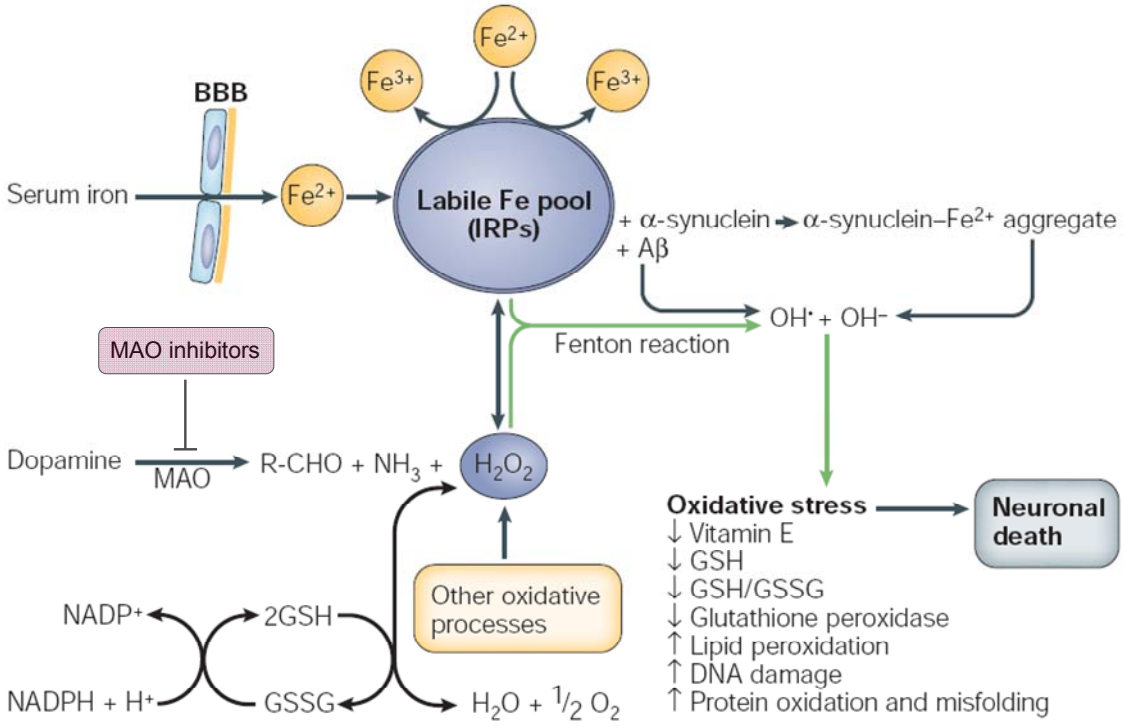

Figure 2-8 Model of neurodegeneration in PD and AD. MAO generates hydrogen peroxide and reactive aldehydes. H_2O_2 participates in the Fenton reaction with ionic iron, producing reactive hydroxyl radicals. The resulting effect is oxidative stress leading to protein oxidation and misfolding, lipid peroxidation and DNA damage. Oxidative stress has consistently been implicated in the pathogenesis of AD, PD and other neurodegenerative diseases. Aβ, amyloid-β; BBB, blood-brain barrier; GSH, reduced glutathione; GSSG, oxidized glutathione; IRPs, iron regulatory proteins. Figure adapted by permission from Macmillan Publisher Ltd: Nature Reviews Neuroscience [260], copyright (2004).

Monoamine oxidases occur as two subtypes, MAO A and MAO B, that can be distinguished by pharmacological and biochemical characteristics [307]. Both are encoded by genes on the X chromosome and share 70% identity in amino acid sequence [301]. The ratio of MAO A:B is specific to tissues [308,309] and cell types [310].

Brain MAO A is expressed in catecholaminergic neurons and glia cells [311-313] where it catalyzes the oxidation of serotonin and norepinephrine. It is selectively inhibited by clorgyline [314] and has long been implicated in the etiology and treatment of depression and anxiety disorders [315,316].

Brain MAO B occurs primarily in serotonergic neurons, and also in glia cells [310]. MAO B exhibits high affinity for phenylethylamine and benzylamine, and is selectively

inhibited by L-deprenyl [317,318]. In clinical practice, selective inhibitors of MAO B are routinely used to alleviate symptoms, or to slow the progression of Parkinson's disease [319,320]. MAO B inhibitors may also prove beneficial in managing Alzheimer's disease or other neurodegenerative disorders [321-325].

Among the naturally occurring polyphenolic inhibitors of MAO A and B that have been identified *in vitro* count (+)-catechin, (−)-epicatechin, quercetin and resveratrol [307,326,327]. However, little is known about the effects of anthocyanins and proanthocyanidins on MAO enzymes.

2.3.2.2.2.5 Induction of hypoxia inducible factor (HIF) expression

A further strategy in disease prevention by polyphenolics addresses brain iron homeostasis. While the prevailing view is that iron chelating anthocyanins and related flavonoids prevent oxidative injury by suppressing Fenton chemistry and the formation of highly reactive hydroxyl radicals, recent data suggest a key role for the hypoxia inducible factors [273,328]. HIFs control the expression of genes involved in diverse processes such as angiogenesis, vascular tone, metal transport, glycolysis, mitochondrial function, cell growth and survival, emphasizing HIF's central involvement in oxygen homeostasis [329]. The heterodimer HIF is a transcription factor composed of HIF-α and HIF-β subunits [330]. Three distinct and non-redundant HIF-α subunits [HIF-1α, HIF-2α/EPAS1, and HIF-3α], as well as three HIF-β subunits [HIF-1β/ARNT1, ARNT2, and ARNT3] are currently known [331], with both HIF-α and ARNT subunits being members of the basic helix-loop-helix Per/ARNT/Sim (PAS) family of DNA binding proteins [332]. While the β subunit is constitutively and stably expressed, expression of the α subunits is contingent on hypoxic conditions within the cell [333]. Under normoxia, HIF-1α subunits are hydroxylated [334] at specific proline residues (positions 402 and 564) [335] and at asparagine 803 [336], whereas HIF-2α is hydroxylated at proline 531 [337]. Hydroxylation of specific amino acids in proteins is an enzyme catalyzed posttranslational modification that can lead to changes in protein stability and interactions between proteins. The group of enzymes that can catalyze HIF-α hydroxylation reaction are the dioxygenases prolyl 4-hydroxylase (PHD) [335] and factor inhibiting HIF-1 (FIH-1). Upon hydroxylation, HIF-α protein is rapidly degraded via an ubiquitination mechanism involving the Hippel-Lindau tumor suppressor protein, and HIF-mediated transcription is prevented [334].

This process may explain a decrease in expression of HIF-dependent cell survival genes in neurodegenerative disease [338]. Moreover, studies on the expression of HIF and its target genes in the adult rodent brain have shown that after focal cerebral ischemia, accompanied by shortage of oxygen, mRNAs encoding HIF-1α, HIF-2α and

downstream genes are up-regulated in the areas around the infarction [331,339-341]. As HIF is at the center of adaptive responses to ischemic and oxidative stress, the regulation of HIF hydroxylation becomes a viable strategy for engaging its homeostatic functions in a host of tissues, particularly the brain [335]. Treatment of isolated neurons and animals with PHD inhibitors may prevent degradation of HIF and may lead to an increase in the expression levels of target genes downstream of HIF [342]. As many HIF-1- or HIF-2-regulated genes, e.g. erythropoietin (EPO), vascular endothelial growth factor (VEGF) and fibroblast growth factor receptor-1 (FGFR-1), prevent oxidative stress-induced death [343-345], HIF-dependent gene expression may provide resistance to oxidative stress and neuronal damage. Importantly, HIF-1 and HIF-2 each appear to regulate a distinct subset of target genes [346,347].

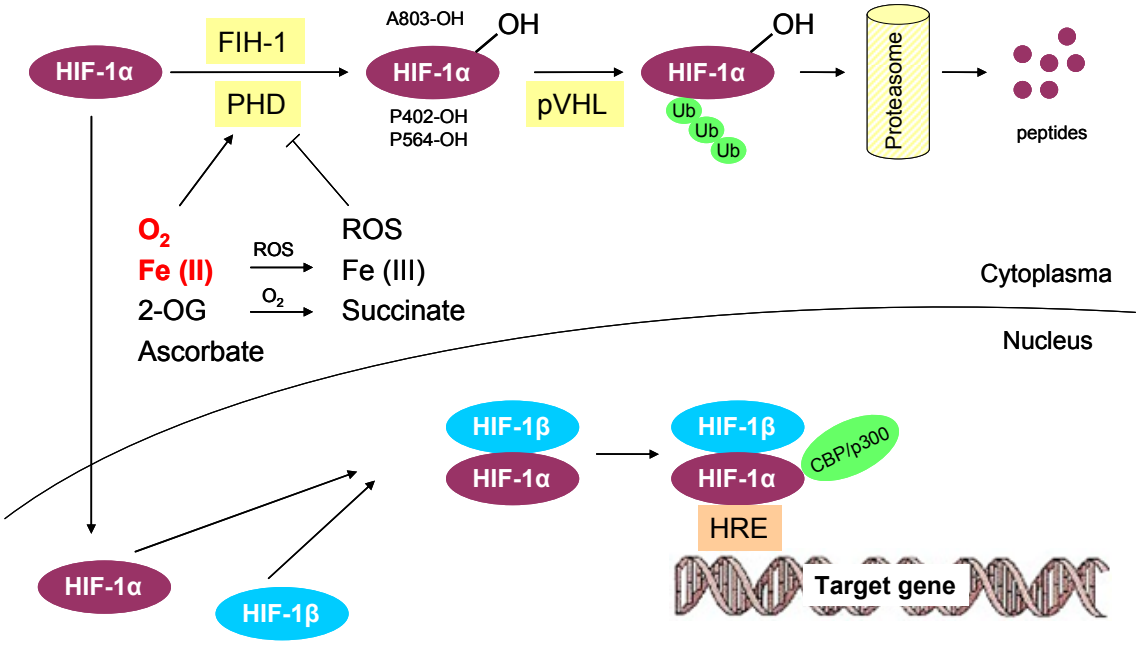

Figure 2-9 Model of HIF-1 pathways: Determined by several factors, HIF-1α may become hydroxylated and degraded via an ubiquitination mechanism in cytoplasma or may form a HIF heterodimer in the nucleus, promoting expression of HIF-dependent target genes. CBP/p300, transcriptional coactivator; FIH-1, factor inhibiting HIF-1; HRE, hypoxia responsive element; 2-OG, 2-oxoglutarate; PHD, prolyl 4-hydroxylase; pVHL, von Hippel-Lindau tumor suppressor; Ub, ubiquitin [335,337,342,348].

In addition to oxygen, ascorbate and 2-oxoglutarate, Fe^{2+} has been shown to enhance PHD and FIH-1 activity [335,336]. The ability of iron to regulate PHD and FIH-1 activity suggests that activity of these dioxygenases can be modulated under conditions of iron deficiency [335]. Ion chelators may stabilize HIF-1*α*, which would heterodimerize with its partner HIF-1β in the nucleus, bind to a hypoxia-responsive element in regulatory genes, and then transactivate the expression of established protective genes [338]. The inhibition of PHD activity in immortalized cells and primary neurons upon treatment

with iron chelators such as desferrioxamine (DFO) indicates that these enzymes rely on the cellular labile iron pool for their activity [342,349]. In addition to causing HIF-1α upregulation, preconditioning with DFO 24 h before hypoxia-ischemia afforded brain protection in neonatal rats [350]. DFO also induced robust tolerance against focal cerebral ischemia in adult mice and rats and increased HIF-1 DNA binding and EPO transactivation [351]). The role of iron chelation in neuroprotective transcriptional pathways thus warrants further research into inhibiting HIF prolyl hydroxylases and activating HIF under conditions of normoxia, and into augmenting HIF activation under conditions of ischemia [352]. Anthocyanins' iron-chelating properties offer a rationale for investigating HIF-1α and HIF-2α induction as a further pathway in neuroprotection.

2.3.2.2.2.6 Proteasome inhibition

The ubiquitin-proteasome system is the major non-lysosomal pathway of proteolysis in human cells and plays a key role in maintaining cellular homeostasis [353]. Specifically, proteasome activity controls the degradation of cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors [354], transcription factors, such as nuclear factor κB and its inhibitors (IκB) [354,355], tumor suppressor proteins [354], misfolded and damaged proteins [356,357] and foreign antigens, among others [358]. As a result of these functionalities, the proteasome is closely implicated in signal transduction, development, cell cycle progression, apoptosis and cancer [359], antigen processing and immune response [360], protection from oxidative stress [361] and inflammation [354,355]. There is little doubt that the proteasome pathway plays a role in diseases associated with oxidative stress and inflammation [362,363] such as brain ischemia, Alzheimer's disease and Parkinson's disease. The identification of novel proteasome inhibitors may therefore open new windows of opportunity in prevention and treatment.

Mammalian cells feature two large proteolytic complexes [364]. The larger structure is the 26S complex (2,000 kDa) that selectively degrades ubiquitinated proteins by an ATP-dependent process [365] and the smaller one is the 20S proteasome (700 to 900 kDa) [366]. The latter degrades peptides independently of ATP and their conjugation to ubiquitin [367,368].

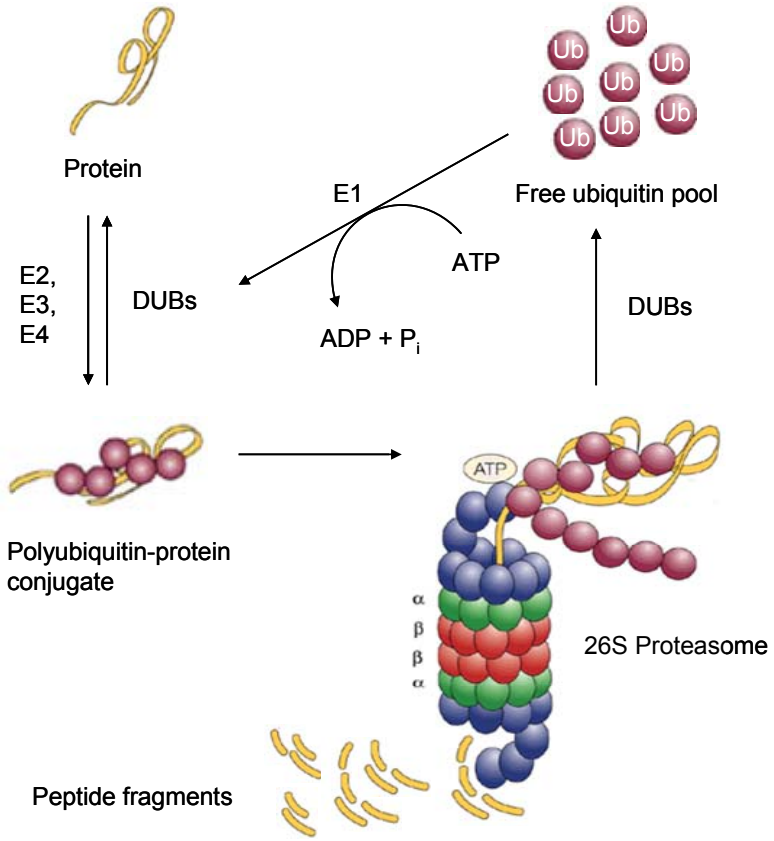

Figure 2-10 The cytoplasmic ubiquitin proteasome system. Ubiquitin (Ub) is activated in an ATP-dependent reaction by the E1 ubiquitin-activating enzyme and is appended to target proteins by the successive action of E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases, and E4 chain elongation factors. Conjugation of multiubiquitin chains targets proteins for degradation by the 26S proteasome, whereas deubiquitinating enzymes (DUBs) oppose ubiquitination by cleaving ubiquitin molecules from targeted proteins and maintain cellular pools of free ubiquitin by recycling ubiquitin monomers. ADP, adenosine diphosphate; ATP, adenosine triphosphate. Figure adapted from [369,370].

In eukaryotic cells, the physiologically relevant form of the proteasome is the 26S complex [371], which is present both in the cytoplasma and the nucleus of all cells [364]. The eukaryotic proteasome, a multicatalytic protease, is characterized by distinct specificities for short synthetic peptides: a “chymotrypsin-like” activity which cleaves after large hydrophobic residues, a “tryptsin-like” activity, which cleaves after basic residues, and a “postglutamyl” hydrolyzing activity with a preference for acidic residues [372]. Two additional specificities have been identified in mammalian proteasomes, one for cleaving after branched chain residues and another for cleavage between small neutral amino acids [373].

While apple and grape extracts have already been described as proteasome inhibitors [374] and inhibition of the chymotrypsin-like proteasome activity in human cells has been reported for various flavonoids [375,376], similar properties of anthocyanins have not been addressed to date.

2.3.2.2.2.7 Phospholipase A_2 inhibition

Phospholipases A_2 count among further potential effectors of neuroprotection. PLA_2s form a superfamily of esterases that specifically cleave the acyl ester bond at the sn-2 position of membrane phospholipids, generating free fatty acids and lysophospholipids [377] (figure 2-11).

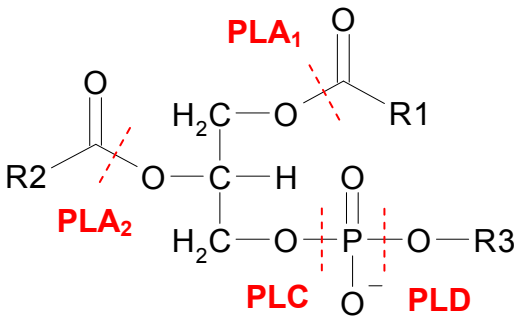

Figure 2-11 General structure of a glycerophospholipid-type phospholipid with cleavage sites for phospholipases A_1, A_2, C and D indicated as dashed lines. R1, R2: fatty acids; R3: choline, ethanolamine, serine or inositol; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D.

These hydrolases are involved in a complex network of signalling pathways, linking receptor agonists, oxidants, and proinflammatory cytokines to the release of arachidonic acid and to eicosanoid synthesis [378]. Eicosanoids include prostaglandins, thromboxanes, prostacyclins, and leukotrienes [379], which act as inflammatory mediators. Moreover, oxidative metabolism of arachidonic acid and disruption of the mitochondrial respiratory chain, mediated by phospholipase A_2 cardiolipin hydrolysis, may contribute to the generation of ROS and oxidative stress [380].

PLA_2s may be grouped into at least three major classes, Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) [381], which are expressed in the central nervous system [378]. Of these, $sPLA_2$ is a major contributor to the excessive production of arachidonic acid in inflammatory conditions [382] and comprises the 14 kDa ‘‘group V’’ PLA_2 with high affinity for phosphatidylcholine-rich plasma membranes [383]. In the mammalian brain, group V $sPLA_2$ is found primarily in cortical neurons [384] and in the hippocampus [385]. Inhibitors of PLA_2 hold promise in the treatment of brain disorders that are associated with oxidative stress, changes in phospholipid metabolism, accumulation of lipid peroxides, and inflammation, e.g. ischemia, multiple sclerosis, epilepsy, and Alzheimer’s disease [386]. Modulation of antioxidant and antiinflammatory activities by

PLA_2 [387], suggests that PLA_2 may be targeted by berry constituents with neuroprotective effects.

2.4 Toxicology and safety

2.4.1 Legal classification and EU safety assessment strategies

When advocating health promoting effects of anthocyanins and proanthocyanidins, it is a major requirement that oral administration of these substances is safe. For a long time, foods from plant origin such as berry fruits, grapes and cocoa have been the only sources of dietary anthocyanins and proanthocyanidins. However, in the past few years, many food bioactive constituents have been commercialized in the form of dietary supplements and functional foods containing berry extracts or grape seed proanthocyanidins.

According to the European Commission, food supplements are concentrated sources of nutrients or other substances with a nutritional or physiological effect, whose purpose is to supplement the normal diet. Food supplements are marketed 'in dose' form, for example as pills, tablets, capsules or liquids in measured doses [388]. In terms of functional food, there is no official definition. One view is that any food is indeed functional because it provides nutrients and has a physiological effect. Others maintain that only fortified, enriched or enhanced foods that confer a health benefit beyond basic nutrition should be considered as functional. Most definitions also suggest that a functional food should be, or look like, a traditional food and must be part of our normal diet. According to a working definition proposed by the European Community (EC) Concerted Action on Functional Food Science in Europe (FUFOSE), functional food describes a food that benefits one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern and is not a pill, a capsule or any form of dietary supplement [389].

Although promising effects on human health may argue in favor of dietary supplements and functional foods, growing evidence questions the beneficial value of dietary supplements such as antioxidant vitamin pills in generally well-nourished populations [390] and underscores the possibility that antioxidant supplements could have unintended consequences for human health.

As a basis for toxicological assessments of plant extracts as active principles of dietary supplements and functional foods, distinct legal classifications of these preparations are required within the European Union. Currently, the use of botanicals and botanical preparations in food is regulated under the General Food Law [391], which attributes the primary legal responsibility for the safety of the products placed on the market to business operators [392]. Due to legal loopholes and broad scopes of interpretation, presently no distinct regulations exist with respect to concentrations of plant extracts to be allowed in dietary supplements and functional foods. For dietary supplements, the European Community Directive 46/2002/EC [393] only regulates the use of vitamins and minerals, while specific rules concerning other substances with physiological effects will be laid down at a later stage. However, the directive notes that “there is a wide range of nutrients and other ingredients that might be present in food supplements including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and various plants and herbal extracts”.

In consequence, prior to enactment of further community regulations, the present national food laws need to be considered in terms of dietary supplements and functional foods. In accordance with EC law, the German Regulation on Food Supplements [394] only classifies vitamins and minerals as nutrients and additives for dietary supplements. Thus, no generally valid rules exist regarding other substances. The situation is similar for functional foods, e.g. foods enriched with berry extracts. In both cases, a major challenge is posed by classification of plant extracts either as conventional foods or as food additives, which currently still requires an individual decision in every single case. According to § 2 (3) of the German Food and Feed Code [395], substances that are usually not consumed as foods by themselves and that are not used as characteristic food ingredients either, are considered equivalents of food additives if they lack a nutritive value and are added for other than technological reasons, e.g. for physiological purposes. Plant extracts for use in functional foods may be regarded as food additive equivalents according to this definition. However, when such extracts are considered for use as part of a dietary supplement, additional limitations must be taken into account. Generally, the classification will depend on whether the extract preparation can preserve the food character, e.g. a typical odor or taste, and also the relative concentrations of constituents present in the original food item. Should plant extracts be added to supplements at concentrations that consumers would not usually incorporate by consuming the original source, restrictions are imposed. At present, the existing positive list of authorized food additives limits the use of anthocyanins to coloring purposes and does not include proanthocyanidins or standardized extracts from berry fruits.

Future legal regulations may include concentration limits and more detailed specifications of bioactive substances in functional foods and dietary supplements. Initial EC-wide progressions are sketched out in Regulation 1925/2006/EC [396], introducing planned proceedings with respect to substances or ingredients "…added to foods or in the manufacture of foods under conditions that would result in the ingestion of amounts of this substance greatly exceeding those reasonably expected to be ingested…" Based on assessments of available information, the Commission plans to include substances in the annex of the above regulation; notably, in part A, substances/ingredients to be prohibited in foods, in part B, compounds to be allowed in foods under certain conditions and in part C, possibly harmful substances with respect to human health. Category C may include substances causing negative effects only under certain circumstances, e.g. when consumed in combination with certain drugs. However, pending the release of final EC regulations, safety evaluations of all extracts to be marketed will depend on a classification by (a) food, including food supplement, or medicine status, (b) novel food status according to regulation EC 258/97 [397] or (c) food additive status.

To facilitate toxicological assessments, the European Food Safety Authority (EFSA) recently published a "Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements" [392]. In this text, EFSA's Scientific Committee provides guidance on the scientific data needed to carry out a safety evaluation of a botanical or a botanical preparation. Using a two-level tiered scientific approach, safety assessment is meant to build on the available knowledge for a given botanical and the substance(s) it contains (level A). This information may then be complemented by newly generated data (level B) (figure 2-12).

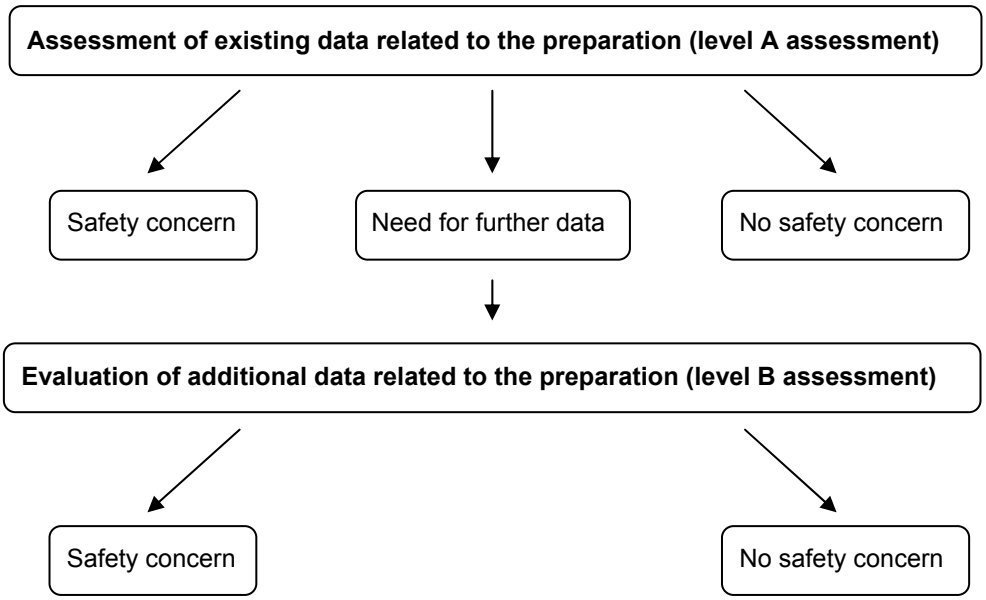

Figure 2-12 Proposed tiered approach for the safety assessment of botanicals and botanical preparations. Figure adapted from [392].

Moreover, the EFSA has compiled the available information on a large number of botanicals which may be of health concern when used in food or food supplements [398]. The resulting compendium, which will be regularly updated, is intended to assist manufacturers and food safety authorities. While neither anthocyanins nor proanthocyanidins are listed as chemicals of concern in the compendium up to date, they cannot automatically be considered devoid of hazards for human health. As it is not the objective of the EFSA to produce a list of safe botanicals and botanical preparations intended for food supplement use, the compendium may only serve as an aid to assess the safety of botanical ingredients.

In summary, legal regulations regarding plant extracts are still being developed and a range of restrictions may be applied to marketing such products. Current guidelines for toxicological assessments highlight a growing awareness of extracts' safety issues. Despite these efforts, the market for dietary supplements has exploded and many products contain berry extracts or proanthocyanidins as active principles. An improved understanding of the respective functionalities can make a significant contribution to enhancing the safety of these agents.

2.4.2 Safety assessment

Safety assessment of plant extracts and isolated compounds is dependent on the level of evidence available from studies of bioactivity. Due to the many constraints in clinical research, most scientific evidence derives from *in vitro* assays and animal testings,

despite their limitations [399]. Common toxicological investigations of candidate compounds in animals include absorption, distribution, metabolism and excretion (ADME) characteristics, acute, subchronic (90 days) and chronic toxicities, covering cancerogenesis, reproductive toxicity, and genotoxicity issues among others.

For non-carcinogens, a so-called no-observed-adverse-effect level or NOAEL can be determined under the assumption of a threshold in dose response [400]. The most common approach to estimating the NOAEL from animal data relies on safety factors. Traditionally, many international regulatory bodies apply a safety factor of 10 when extrapolating animal data to humans. These animal NOAELs that are expressed per body weight are divided by the safety factor to derive the “safe” level of human exposure. In order to account for interindividual variability, a further safety factor of 10 is frequently applied [401,402]. If toxicity data from human studies are available, these take priority over animal data. In such cases, a safety factor of 10 rather than 100 is judged appropriate [401]. Further factors may be applied, e.g. for extrapolation of short-term to long-term studies [399] and to correct for possible synergistic effects of multiple compounds in the human body [403]. Final safety factors for extrapolations of data to humans may thus range between 10 and 10,000, depending on the situation. Greater factors also imply that the information is very imprecise and may not allow reliable conclusions on risks for human health [399].

The safe human exposure is usually termed “acceptable” for food additives or “tolerable” for food contaminants. An approach based on a daily intake yields the prevailing terms “acceptable daily intake” (ADI) and “tolerable daily intake” (TDI). According to World Health Organization / International Programme on Chemical Safety (WHO / IPCS) criteria, the ADI is defined as the estimated maximum amount of an agent, expressed on a body mass basis, to which individuals in a (sub)population may be exposed daily over their lifetimes without appreciable health risk. TDI is used analogous to ADI for agents that are not deliberately added, such as contaminants in food [404]. Regarding food ingredients, including anthocyanins and proanthocyanidins, and food contaminants, the Joint Food and Agriculture Organization (FAO) / WHO Expert Committee on Food Additives (JECFA) evaluates risk potentials and calculates ADI values [405].

2.4.2.1 Anthocyanin toxicity

The number of toxicological studies on anthocyanins is limited [406]. An ADI for anthocyanins from grape skin was determined earlier at 0 to 2.5 mg/kg body weight

(bw)/day based on short-term toxicity studies of grape-skin extract. For anthocyanins in general, no ADI was allocated due to the lack of sufficient toxicological data [407].

For cyanidin and delphinidin no signs of mutagenicity were reported in the Ames assay system using five different bacterial strains *in vitro* [408]. *In vivo*, an extremely low acute oral toxicity of mixed anthocyanin extract from currants, blueberries and elderberries (cyanidin, delphinidin, petunidin) is illustrated by mouse and rat LD_{50} values greater than 25 and 20 g/kg bw, respectively [409]. However, some minor side effects have been observed *in vivo*. At oral doses of 500 mg/kg bw anthocyanins may produce a sedative effect in mice. Intravenous administration of 100 to 200 mg/kg bw anthocyanins may elicit transient hypotension and a decrease in respiratory amplitude. Finally, at 25 mg/kg bw, diuretic effects were reported [409].

Subchronic toxicity studies over a period of three months did not show any overt signs of toxicity in animals, e.g. in rats given mixed anthocyanin extract at oral doses of 6 g/day [409]. Using anthocyanins from purple corn, NOAELs of 0.94 and 1.02 g/kg bw/day for male and female rats were obtained, respectively [410]. No adverse subchronic effects emerged in beagle dogs on a diet containing 7.5 or 15% grape color powder [411]. Moreover, anthocyanin extract from currants, blueberries and elderberries was reported not to be teratogenic in rats, mice or rabbits when given at oral dose levels of 1.5, 3 or 9 g/kg bw over three successive generations [409].

So far, about 300 prospective studies have been conducted in humans with respect to bioavailability and health promoting effects, of which the majority refers to single-dose and short-term regimens. Anthocyanins have been administered at doses ranging up to several g per day. At single oral doses of 1 to 2.4 g anthocyanins, no adverse effects have been recorded [86,88,106,183,184,412]. In 7-day studies, 1.4 g/day and 2.7 g/day were well tolerated [413,414], and no signs of subchronic toxicity were observed in humans consuming 500 mg anthocyanins from elderberry extract per day for 12 weeks [415]. With regard to safety of a standardized anthocyanin extract, reference has been made to a post-marketing surveillance study on 2,295 subjects, of which the majority consumed 115 mg anthocyanins per day for one to two months. In most cases, anthocyanin extract was reportedly tolerated well or very well at a 4% rate of minor side effects concerning the gastrointestinal tract, skin, cutaneous annexes and the nervous system [416]. It should be noted, however, that the authors have not published this safety information.

In summary, studies performed with isolated compounds are rare and available data on the toxicity of anthocyanin preparations are incomplete. Of those clinical trials that have monitored adverse effects of anthocyanins only a fraction fulfills current standards of randomization or blinding, and many were uncontrolled.

2.4.2.2 Proanthocyanidin toxicity

According to *in vitro* evidence, grape seed proanthocyanidins are neither mutagenic [417], nor do they cause chromosomal damage [209].

From acute oral toxicity studies, the GSPE LD_{50} has been estimated greater 5 g/kg rat bw, corresponding to a dosage of about 300 g in a human with 60 kg bw [417,418]. For rats, a NOAEL of 2 g GSPE/kg bw was observed for systemic toxicity [418] and a NOAEL of 4 g/kg bw was reported for oral toxicity [419].

From subchronic (three months') oral toxicity studies, NOAELs of approximately 1.78 and 2.15 g/kg bw/day (2.5% diet) were derived for male and female rats, respectively [420]. A 2% GSPE diet reportedly decreased serum iron levels of animals, but did not otherwise cause any adverse effects [421]. Due to their *o*-dihydroxyphenyl groups, PAs are excellent chelators of iron (III) and known to form stable complexes with metal ions [47]. This property may be an issue in developing countries where the majority of the population relies on diets rich in beans for protein nitrogen [422]. Long-term (six months') administration of GSPE at 500 mg/kg bw/day to mice failed to produce any signs of chronic toxicity [418].

In humans, no subchronic toxicity was noted following daily consumption of 400 mg proanthocyanidins from grape seeds [198]. During long-term administration of GSPE at 1 g/day and 300 mg/day for six weeks and six months, respectively, no side effects were reported [133,423].

However, unresolved questions remain in that the monitoring of adverse effects has varied and available data on proanthocyanidin toxicity are mostly based on GSPE administration rather than on isolated proanthocyanidins.

2.4.3 Interactions with xenobiotic metabolism

Although available studies on acute, subchronic and chronic toxicities of anthocyanins and proanthocyanidins have not shown any serious side effects, we cannot conclude that consumption of anthocyanin- and proanthocyanidin-rich products is generally safe. Since flavonoids may interact with metabolism of other food compounds and drugs, changes in xenobiotics' uptake, excretion and metabolism rates may cause adverse effects and toxicities. Possible hazards to human health are not predictable, especially if various dietary supplements are consumed in combination with pharmaceuticals. An example of known interactions is given by the ability of phytic acid to increase anthocyanins' absorption and urinary excretion in rats and humans [100]. Conversely, an anthocyanin-rich Hibiscus beverage may reduce plasma bioavailability of the antimalaria drug chloroquine [424]. These observations highlight the importance of

studying in more detail those metabolomic pathways that may be utilized by both berry constituents and prescription drugs.

2.4.3.1 Cytochrome P450 enzymes

Cytochrome P450 enzymes play key roles in the detoxification of xenobiotics, including toxins, carcinogens, and drugs [155]. Typically, most lipophilic drugs and chemicals that enter the body are biotransformed into inactive, more hydrophilic, readily excretable metabolites [425]. This transformation can be divided into two steps: phase I and phase II metabolism [426]. Phase I metabolism usually results in the introduction, modification or liberation of functional groups via oxidation, reduction, isomerisation or hydrolysis reactions [427]. In phase II metabolism, conjugation of functional groups occurs with hydrophilic biochemicals of endogenous origin [426], so as to render the respective metabolites inactive and more readily excreted [425].

Cytochrome P450 enzymes are active during phase I and represent a large family of microsomal heme-containing monooxygenase isoenzymes. They are primarily expressed on smooth endoplasmic reticulum membranes by liver hepatocytes and by cells along the intestinal tract mucosal surface [428], where they act as catalyzers of hydroxylations and other oxidation reactions [155]. Cytochrome P450 enzymes are involved in the detoxification of a wide variety of xenobiotics such as drugs, biogenic amines from food sources, environmental toxins, and chemical carcinogens. Other functionalities comprise the oxidation of steroids, fatty acids, prostaglandins, leukotrienes, and fat-soluble vitamins [429-431]. In addition, they play key roles in activating many toxins and procarcinogens that are turned into powerful alkylating substances [155,432].

With regard to dietary compounds, cytochrome P450 enzymes have been implicated in numerous food-drug interactions [433]. The opportunity for such interactions is an everyday occurrence, of which grapefruit juice provides a prominent example. Coadministration of grapefruit juice with many therapeutic agents is known to increase the oral bioavailability of common drugs by altering presystemic metabolism, particularly in the intestine [434]. Interactions with enzyme inhibitors have been shown to affect both pharmacokinetic and pharmacodynamic parameters of drugs [435,436], and to augment drug toxicity. Recognition of food–drug interactions has raised concerns about risks that may be posed by functional foods, and specifically, by food enriched with secondary plant metabolites.

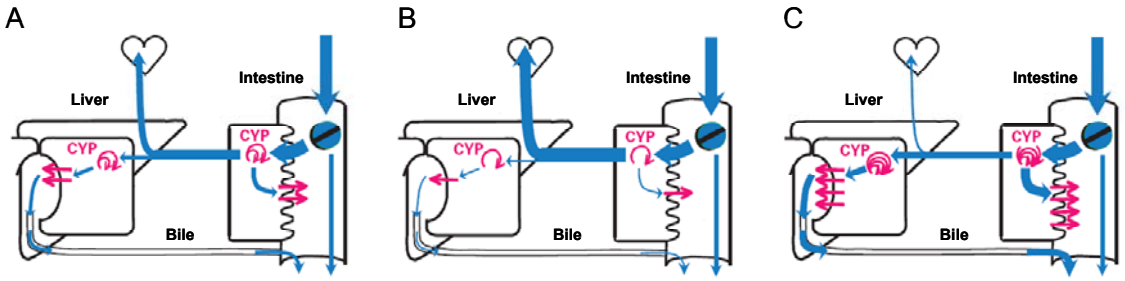

Figure 2-13 Model of Cytochrome P450 (CYP) enzyme-mediated xenobiotic metabolism in the liver and intestine (A), and impact of cytochrome P450 inhibition (B) and induction (C) on xenobiotic pharmacokinetics. Figure adapted from [437] and used with kind permission of Schweizerischer Ärzteverlag AG (http://www.medicalforum.ch).

In isolated human liver microsomes, grapefruit furanocoumarins acted as inhibitors of isoenzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 [438]. With respect to dietary anthocyanins and proanthocyanidins, weak inhibitory interactions of anthocyanins and aglycones have emerged with CYP2C19 [439], CYP2C9 [440] and CYP1A2 [441], and of GSPE with cytochrome CYP2E1. In support of these data, a 40% inhibition of aniline hydroxylation was observed in rats receiving GSPE at 100 mg/kg bw/day for four weeks [442]. As CYP2C19, CYP2C9 and CYP2E1 may oxidize environmental chemicals, such as nitrosamines, acrylamide, benzo-[a]-pyrene, and various organic solvents, an improved understanding of enzyme inhibitors may also serve to prevent the formation of carcinogens.

2.4.3.1.1 Cytochrome P450 3A4

The CYP3A subfamily comprises 30% of the total liver cytochrome P450 enzyme pool in humans [443] and an estimated 70% of CYP protein in the small intestinal epithelium [444]. 60% of all drugs metabolized are targeted by the 3A4 isoenzyme [445].

Coadministration of multiple CYP3A4 substrates, inducers, or inhibitors, including compounds from food sources, may result in adverse effects of commonly prescribed drugs [435,436]. A prominent example of food–drug interactions mediated by CYP3A4 is provided by the inhibitory effects of grapefruit juice on presystemic metabolism, particularly in the intestine [434]. Constituents of grapefruit juice with CYP3A4 inhibitory activities include furanocoumarins and the flavonoids naringin, quercetin, and kaempferol [438]. With respect to dietary anthocyanin sources, *in vitro* CYP3A inhibitory potential has been described for black raspberry juice, wild grape juice, black mulberry juice [446], pomegranate juice [447], and red wine [448], and has been suggested for cranberry juice [449]. With drug safety in mind, more detailed investigations of berry polyphenols are urgently warranted.

2.4.3.1.2 Cytochrome P450 2D6

Within the cytochrome P450 superfamily, the cytochrome P450 2D6 isoenzyme plays a key role in the metabolism of centrally acting drugs [450]. These include many neuroleptics [451], selective serotonin reuptake inhibitors (SSRIs) [452], selective norepinephrine reuptake inhibitors (SNRIs) [453] and tricyclic antidepressants (TCAs) [454] that are metabolised via hydroxylation [455], demethylation [456] or dealkylation reactions [431]. The human brain's CYP2D6 expression pattern maps to the dopaminergic system [457], where CYP2D6 converts the endogenous substrate tyramine, which is formed from tyrosine or phenylalanine, to dopamine. This implies that inhibitors may alter tyrosine and dopamine levels via brain CYP2D6.

In an estimated 10% of the general population, CYP2D6 substrate metabolism may be seriously compromised owing to an innate deficiency in enzymatic activity [458,459]. CYP2D6 inhibitors from food sources may also interfere with the metabolism of psychoactive drugs and limit the use of medication. Furanocoumarins [438], polyphenol-rich green tea and grape seed extracts [460], plus the flavonoids naringenin, vitexin and quercetin [461], have already been identified as CYP2D6 inhibitors. However, anthocyanins' ability to interfere with the metabolism of centrally acting drugs via inhibition of CYP2D6 has not been examined.

3 Results and discussion

3.1 Anthocyanin bioavailability: transport mechanisms

To date, limited data are available on transport mechanisms that may control anthocyanin absorption and brain bioavailability. In order to determine whether anthocyanins are substrates for ABC transporters, and whether anthocyanins may interfere with the transport of other substrates, 16 anthocyanidins and anthocyanins were tested for *in vitro* effects on the human efflux transporters BCRP and MDR1. Interactions were studied in dye extrusion and ATPase assays as well as in vesicular transport assays for BCRP. In combination, these assays give insight into different types of transporter interaction and specify substances' affinities for the ABC transporters under study.

None of the test compounds showed any affinity for either the BCRP or the MDR1 transporter in dye efflux assays. Passive permeability is a prerequisite for eliciting a response in dye extrusion assays as has previously been shown for other compounds [462,463]. The absence of effects in the calcein acetoxymethyl ester (calcein-AM) and Hoechst 33342 assays suggests that anthocyanins and their aglycones cannot cross the cell membrane passively and, therefore, will not reach the substrate binding site of the transporter.

For all other BCRP and MDR1 assays performed, calculated reaction parameters are shown in table 3-1.

Table 3-1 Reaction parameters derived from MDR1 plus BCRP ATPase and BCRP vesicular transport assays. With kind permission from John Wiley and Sons [464].

Test compound	MDR1 ATPase	BCRP ATPase		BCRP VT
	Inhib [IC_{50}] (eff)	Inhib [IC_{50}] (eff)	Activ [EC_{50}] (eff)	Inhib [IC_{50}] (eff)
Cyanidin	58 (100)	7.6 (100)	- (-)	5 (100)
Delphinidin	104 (100)	87 (100)	- (-)	13 (98)
Malvidin	220 (62)	49 (100)	1.1 (59)	3 (98)
Pelargonidin	83 (100)	76 (79)	- (-)	17 (97)
Peonidin	100 (100)	30 (100)	- (-)	4 (96)
Petunidin	230 (65)	193 (87)	3.3 (125)	5 (100)
Cyanidin-3-glc	- (-)	- (-)	119 (142)	49 (87)
Cyanidin-3-gal	- (-)	- (-)	15 (237)	25 (89)
Cyanidin-3-rut	- (-)	- (35)	- (-)	95 (73)
Cyanidin-3,5-diglc	- (-)	43 (100)	- (-)	83 (76)
Delphinidin-3-glc	- (-)	- (44)	- (-)	47 (93)

Malvidin-3-glc	- (42)	- (35)	- (-)	43 (94)
Malvidin-3-gal	- (24)	- (14)	6.3 (73)	31 (88)
Malvidin-3,5-diglc	- (-)	- (-)	12 (87)	23 (81)
Pelargonidin-3,5-diglc	- (14)	- (24)	- (-)	130 (66)
Peonidin-3-glc	- (20)	- (-)	45 (133)	24 (93)

Half-maximal inhibition and activation values are displayed in µM. Efficacies (eff) are given in brackets at the highest anthocyanidin/anthocyanin concentration tested. "-" no effect observed; activ, activation; inhib, inhibition; VT, vesicular transport.

In BCRP ATPase assays, baseline ATPase activity was stimulated by seven anthocyanidins and their glycosides, with half-maximal activation in the low micromolar range for two anthocyanidins. The presence or absence of a sugar moiety failed to predict half-maximal ATPase activation values in this assay ($P = 0.1504$, $t = 1.78$, df = 4). For four flavonoids, stimulation of baseline ATPase activity exceeded 100% as defined by the reference activator substrate sulfasalazine. As this was in part a non-specific effect that was also observed in the defBCRP negative control for cyanidin-3-gal and cyanidin-3-glc, the net activation may be less pronounced for these compounds. In contrast, petunidin and peonidin-3-glc emerged as genuine stimulants of BCRP ATPase activity that led to a higher level of activation than was achieved with 10 µM sulfasalazine. Stimulation of ATPase activity by substrates is considered a direct correlate of the actual transport process [152,465] and therefore an indicator of substrate functionality. However, stimulation of BCRP- and MDR1-associated ATPase activity may, on occasion, occur independently of substrate transport [466,467]. In other cases, induction of ABC transporter activity has been observed [468]. This appears unlikely for anthocyanins and anthocyanidins, as reporter substrate transport was not increased in the vesicular transport assay.

In addition to some stimulators of BCRP ATPase, potent inhibitors were also identified, with IC_{50} values of cyanidin, peonidin, cyanidin-3,5-diglc and malvidin ranging from 7.6 to 49 µM. With only one exception, cyanidin-3,5-diglc, the absence of sugar moieties was associated with higher levels of BCRP ATPase inhibition, as compared with those achieved by other compounds. Intermediate inhibitors comprised pelargonidin and delphinidin, and minimal inhibition was observed for cyanidin-3-rut, delphinidin-3-glc, malvidin-3-glc, pelargonidin-3,5-diglc, malvidin-3-gal (eff. < 44%). Of the identified inhibitors, delphinidin, cyanidin, malvidin and peonidin inhibited both the maximal ATPase activity after sulfasalazine stimulation, and the baseline ATPase activity at higher concentrations. Interestingly, two compounds, malvidin and petunidin, which stimulated the BCRP ATPase at low concentrations (EC_{50} of 1.1 and 3.3 µM, respectively), displayed bimodal activities, inhibiting sulfasalazine-stimulated ATPase

activity at higher concentrations (IC_{50} = 49 µM and 193 µM, respectively). It cannot be entirely ruled out, however, that these two anthocyanidins are not true inhibitors, but rather slowly transported substrates which compete with the assay substrate for transport.

In contrast to results for BCRP ATPase, the effects of the test compounds on MDR1 ATPase activity were weak. Cyanidin-3-glc, cyanidin-3-gal and delphinidin-3-glc showed weak stimulation of both baseline and verapamil-induced activity (eff. = 35%, 23% and 24% respectively). The latter effect is rarely observed in practice and findings are best explained by nonspecific interactions with the MDR1 transporter, such as stimulation of an endogenous ATPase. For these flavonoids, a slight stimulation of ATPase activity in the beta-gal control membranes was also observed. All tested aglycones reached IC_{50} values from 58 to 230 µM for MDR1 ATPase inhibition, but only cyanidin, pelargonidin, peonidin and delphinidin fully inhibited verapamil-stimulated ATPase activity within the tested concentration range (< 300 µM). Partial and very weak MDR1 inhibition was observed for malvidin, petunidin, malvidin-3-glc, malvidin-3-gal, peonidin-3-glc and pelargonidin-3,5-diglc. Finally, cyanidin-3-rut, cyanidin-3,5-diglc and malvidin-3,5-diglc did not exhibit any measurable inhibitory effects on MDR1 ATPase. Thus, anthocyanidins are either moderate MDR1 inhibitors or slowly transported MDR1 substrates, while even lower levels of inhibition were observed for glycosylated compounds.

In view of the overall poor affinities of these compounds for MDR1, further characterizations by transport assays to rule out non-specific stimulation of ATPase were conducted only for BCRP. Since all test compounds inhibited BCRP-mediated transport of ^{3}H-oestrone-3-sulphate into vesicles dose dependently (far right hand column, table 3-1), vesicular transport assays served to verify affinities of all tested compounds for BCRP. Effects were most pronounced for malvidin, peonidin, petunidin and cyanidin with essentially identical IC_{50} values and full efficacy. The remainder of the compounds tested were less potent with IC_{50} values ranging from 13 µM to 130 µM and efficacies from 98% to 66%. With regard to structural features, results of the vesicular transport assay confirmed significantly higher affinities of anthocyanidins for BCRP (mean IC_{50} = 7.8 µM) when compared with affinities of glycosylated test compounds (mean IC_{50} = 55.0 µM, p = 0.0029, t = 4.05, df = 9). Moreover, none of the tested anthocyanins or aglycones enhanced transport of the reporter substrate. This further supports the hypothesis that the seven compounds activating BCRP ATPase are true BCRP substrates, rather than mere stimulants of BCRP activity.

A variety of dietary polyphenols have been described to interact with BCRP or MDR1 *in vitro*. Of these, the stilbene resveratrol, the flavanones hesperetin [469] and naringenin

[470], the flavones luteolin and chrysin [471], and the flavonols kaempferol and quercetin [472] have emerged as inhibitors of BCRP, among others. The isoflavone genistein is considered a natural substrate that competitively inhibits drug efflux by BCRP [471]. With respect to MDR1, resveratrol [473], hesperetin, naringenin, chrysin [474] the tea catechins epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), plus the anthocyanidin cyanidin [475] have previously displayed inhibitory activities. Regarding bimodal functions, as observed for malvidin and petunidin with respect to BCRP ATPase activity, similar effects have been noted for quercetin and kaempferol on MDR1 activity [474,476]. Polyphenolic MDR1 stimulants include (-)-epicatechin [477] and the flavonol galangin [478].

Owing to the mostly semi-quantitative and heterogenous assays employed in many earlier studies [479], however, a cautious comparison of flavonoids' affinities for BCRP and MDR1 is warranted. Among the consistent findings is the modulatory role of sugar moieties on efflux transporter activity. Thus, for most flavonoids that interacted with either BCRP or MDR1, the corresponding glycosides proved either inactive or less active [471,472,474-476]. In a further parallel to earlier studies on other polyphenols [471,480-482], prominent effects of anthocyanidins on BCRP were noted in this work (figure 3-1). As BCRP ATPase inhibition was up to sevenfold that for MDR1, and substrate-like behavior was limited to BCRP, BCRP-specific functions may be exhibited by anthocyanidins and anthocyanins.

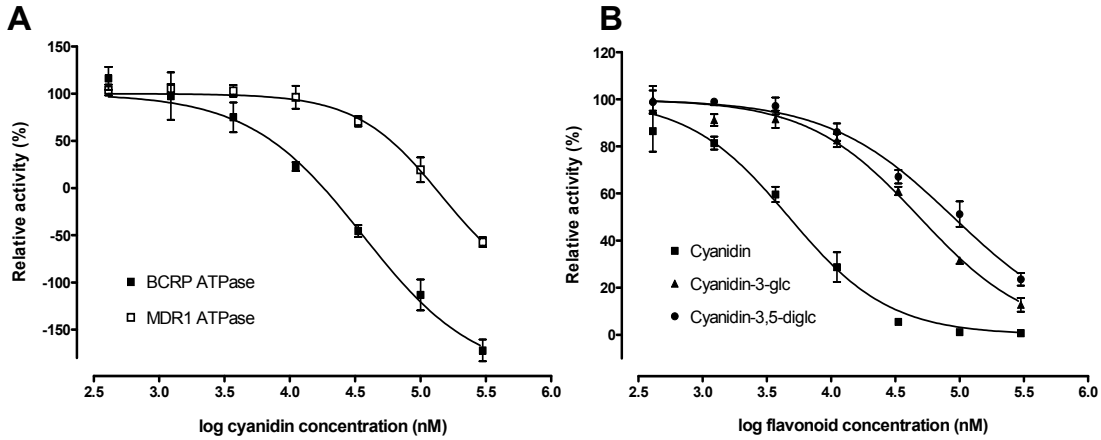

Figure 3-1 Dose-response curves for cyanidin with respect to BCRP and MDR1 ATPase inhibition (A), and for cyanidin, cyanidin-3-glc and cyanidin-3-diglc with respect to inhibition of BCRP vesicular transport (B). BCRP, breast cancer resistance protein; MDR1, multidrug resistance protein 1. With kind permission from John Wiley and Sons [464].

This may be relevant to the intestinal barrier and to brain microvessels where BCRP is expressed at higher levels than MDR1 [483-485]. Despite the difference in transporter

expression, however, the role played by BCRP in substrate efflux at the BBB *in vivo* is less firmly established [486,487].

While substrate-type affinity of anthocyanidins and anthocyanins for BCRP is liable to limit their intestinal absorption and, possibly, brain uptake, the moderate levels of interaction with MDR1 would appear not to be able to reduce bioavailability. On the other hand, substrate-type or inhibitor-type affinities of anthocyanidins, anthocyanins, and other dietary compounds may also facilitate crossing of the intestinal and blood-brain barriers by other anthocyanidins and anthocyanins and thereby may augment berry flavonoids' bioavailability.

However, for anthocyanidin interactions with BCRP and MDR1 transporters to take effect, it is required that anthocyanidins enter intestinal epithelial and brain endothelial cells at micromolar concentrations. Existing data point to relatively high anthocyanin concentrations in the human intestine [86], whereas peak plasma concentrations have been described in the nanomolar range [70,93,488]. Although anthocyanin brain bioavailability is not sufficiently elucidated to date and raises several questions, results from animal studies would appear to rationalize interactions with ABC transporters in brain endothelial cells *in vivo* as these agents rapidly cross the blood-brain barrier [80,83,112-114]. Favorable changes in central nervous parameters of oxidative stress following administration of anthocyanins further corroborate this hypothesis [16] but detailed tissue quantitations of anthocyanins and anthocyanidins have yet to be performed and concentrations in brain endothelial cells still need to be elucidated.

Moreover, estimates on anthocyanin bioavailability may be considered conservative in that analytical challenges are posed by anthocyanin accumulation in tissues as shown for rats [82], by metabolic transformation to molecular structures that are not routinely detected, and by protein binding [115,489,490].

For extrapolations to the *in vivo* situation, functional genetic variation in transporter proteins must also be taken into account as a likely confounder of the functionalities addressed [491-493]. More detailed investigations of anthocyanin and anthocyanidin transporter binding sites and interaction mechanisms are desirable, including studies on reversibility of effects. Possible mechanisms include competitive inhibition or steric blockage of substrate binding to the transporter or ATPase, and allosteric effects on substrate recognition, translocation, dissociation or ATP hydrolysis. Moreover, it cannot be excluded that anthocyanins' interactions with ABC transporters in the human body may be different from those observed during the present *in vitro* testings, since complex food matrices may also exhibit unknown synergistic or quenching effects *in vivo* and since compounds' metabolism needs to be considered as well. Therefore, other areas of future research may include effects of combinations of anthocyanins, as

in standardized extracts, and of anthocyanin degradation products such as phenolic acids on ABC transporters. Finally, direct transport assays and studies on BCRP knockout mice to rationalize this works' assumptions and comparative studies involving other members of the ABC transporter family are warranted.
In summary, results of the present work demonstrate moderate to high affinities of anthocyanins and anthocyanidins for the human efflux transporter BCRP, and moderate to low affinities for MDR1. These results add to our understanding of anthocyanin plasma and brain bioavailability.

Finally, anthocyanins and anthocyanidins may also have potential to alter bioavailability of other compounds. Therefore investigations on interactions of anthocyanins and their aglycones with ABC transporters BCRP and MDR1 add to a better understanding of safety concerns in terms of these berry flavonoids. Regarding polyphenols, the isoflavone genistein is considered a natural substrate that competitively inhibits drug efflux by BCRP [471]. Similarly anthocyanins and anthocyanidins may be able to interfere with transport of other BCRP or MDR1 substrates. Distinct affinities of xenobiotics for BCRP are also discussed for the substrates flavopiridol, mitoxantrone and topotecan [494-496], plus the inhibitory fumitremorgin C analogue Ko143 and tariquidar analogues [497,498]. However, other food constituents may be BCRP substrates as well. In consequence, targeting of BCRP by dietary compounds such as anthocyanins may affect barriers in the placenta [499], secretory organs [500], the digestive tract and the brain [501] with regard to permeability for toxins [502], other dietary compounds [152,503,504] and environmental carcinogens [505]. Moreover, BCRP has been implicated in multidrug resistance of tumors [153,480], and co-determines responsiveness to the treatment of CNS disease [506,507].
Results of the present work suggest that anthocyanidins may alter the pharmacokinetics of above mentioned xenobiotics, especially by competing with and thereby limiting transport of drugs with high affinities for BCRP. These berry flavonoids causing efflux transporter inhibition may on the one hand dismantle protection against toxins [491], or may pose a risk of interactions with xenobiotics and other food ingredients including those deriving from dietary supplements [156,472]. On the other hand, transporter inhibitors can restore chemosensitivity to tumor cells [154,472] and may enhance brain uptake of drugs. As adjuvants to therapy, identified inhibitors thus hold promise in the pharmacotherapy of epilepsy [506-508] and primary brain tumors [147,508,509].
These results put into perspective the potential of berry anthocyanidins to interfere with the transport and the pharmacokinetics of other MDR1 and BCRP substrates. More

detailed toxicity profiles will help to unveil the related benefits and novel dietary or medical applications of anthocyanins. Since bioactive compounds may be ingested regularly and in significant amounts when consumed in form of dietary supplements and functional foods, especially long-term toxicology studies of pure anthocyanins and of anthocyanins embedded in complex matrices are warranted.

3.2 Neuroprotective effects

3.2.1 Effects on monoamine oxidases A and B

In the light of neuroprotective properties described for berry constituents with mechanisms being for the most part still unknown, studies on potential effectors of neuroprotection conferred by anthocyanins, anthocyanidins and proanthocyanidins is in great demand. Considering low brain tissue concentrations anthocyanins reach in animal studies, it is unlikely that direct radical quenching presents their primary mode of action. Since emerging neuroprotective effects of anthocyanins from berry fruits may also be explained by an affinity of these polyphenols for monoamine oxidases [510], MAO isoforms A and B count among novel candidate effectors mediating health effects on the human brain. Therefore 10 anthocyanins', six anthocyanidins', two proanthocyanidins' and seven phenolic metabolites' impact on MAO A and MAO B activities was examined.

Test compounds inhibited MAO A and B enzyme activities in a concentration-dependent manner and exhibited significant differences in IC_{50} values between substance groups (table 3-2, table 3-3, figure 3-2). For MAO A, IC_{50} values indicated strongest inhibition by anthocyanidins (mean 29.2 +/- 4.4 µM SD), followed by anthocyanidin-3-glycosides (mean 36.9 +/- 5.8 µM SD), and anthocyanidin-3,5-diglucosides (mean 97.3 +/- 31.0 µM SD) and finally, by phenolic acids (mean 11,489 +/- 7,022 µM SD). IC_{50} values of proanthocyanidins B1 and B2 could not be calculated as these compounds did not achieve 50%-inhibition at maximum concentrations of 1,000 and 2,000 µM, respectively. The known MAO A inhibitor clorgyline achieved an IC_{50} value of 5.4 nM.

Table 3-2 IC_{50} values calculated for inhibition of MAO A activity by anthocyanidins, anthocyanins, procyanidins, phenolic acids and clorgyline.

Test compound	IC_{50} in µM (95% confidence interval)
Malvidin	22 (21-23)
Pelargonidin	27 (26-28)
Delphinidin-3-glc	29 (29-30)
Cyanidin	30 (29-31)
Peonidin	31 (29-32)
Petunidin	32 (30-33)
Cyanidin-3-rut	33 (32-34)
Cyandin-3-glc	34 (33-35)
Delphinidin	35 (33-37)
Cyanidin-3-gal	36 (35-37)
Peonidin-3-glc	38 (36-40)
Malvidin-3-gal	39 (37-42)
Malvidin-3-glc	48 (45-51)
Malvidin-3,5-diglc	62 (58-65)
Cyanidin-3,5-diglc	113 (108-118)
Pelargonidin-3,5-diglc	117 (113-122)
Procyanidin B1	> 1,000 (-)
Procyanidin B2	> 2,000 (-)
Vanillic acid	3,885 (3,703-4,076)
Syringic acid	4,159 (3,894-4,442)
3-(4-Hydroxyphenyl)propionic acid	5,591 (5,251-5,953)
4-Hydroxybenzoic acid	11,522 (10,906-12,172)
Protocatechuic acid	17,229 (16,287-18,224)
3-Hydroxyphenylacetic acid	17,755 (16,807-18,757)
4-Hydroxyphenylacetic acid	20,282 (19,093-21,545)
Clorgyline	5.4 nM (4.4-6.6 nM)

For MAO B, in turn, IC_{50} values testified to strongest inhibition by anthocyanidins (mean 32.7 +/- 10.5 µM SD), followed by anthocyanidin-3-glycosides (mean 36.8 +/- 5.2 µM SD), and anthocyanidin-3,5-diglucosides (mean 155.7 +/- 84.4 µM SD). Proanthocyanidins B1 and B2 failed to achieve 50%-inhibition at a maximum concentration of 400 µM, while an IC_{50} value of 135 nM was determined for the known MAO B inhibitor R-(-)-deprenyl.

Table 3-3 IC_{50} values calculated for inhibition of MAO B activity by anthocyanidins, anthocyanins, procyanidins, phenolic acids and deprenyl.

Test compound	IC_{50} in µM (95% confidence interval)
Malvidin	19 (18-20)
Peonidin	22 (20-25)
Delphinidin-3-glc	31 (29-34)
Cyanidin	32 (30-33)
Cyanidin-3-glc	33 (30-36)
Malvidin-3-gal	34 (32-37)
Cyanidin-3-rut	35 (34-37)
Malvidin-3-glc	37 (35-39)
Delphinidin	38 (36-41)
Peonidin-3-glc	41 (38-43)
Pelargonidin	43 (40-45)
Petunidin	43 (41-46)
Cyanidin-3-gal	46 (45-48)
Malvidin-3,5-diglc	73 (67-80)
Cyanidin-3,5-diglc	152 (139-166)
Pelargonidin-3,5-diglc	242 (221-265)
Procyanidin B1	> 400 (-)
Procyanidin B2	> 400 (-)
R-(-)-deprenyl	135 nM (119-153 nM)

MAO A inhibition by anthocyanins and anthocyanidins falls within the range of IC_{50} values previously determined for other polyphenols, e.g. quercetin (50 µM), *trans*-resveratrol (17.4 µM), apigenin (1.7 µM), and kaempferol (0.7 µM) [327,511,512]. Likewise, for MAO B, findings are in close agreement with published IC_{50} values for quercetin (90 µM), (+)-catechin (88.6 µM), (−)-epicatechin (58.9 µM), *trans*-resveratrol (30.8 µM) and apigenin (12.8 µM) [307,327,511]. Owing to methodological issues, e.g. different sources of MAO enzymes, a direct comparison of results on compounds investigated with above flavonoid IC_{50} values must be conducted cautiously. When compared to known selective inhibitors, MAO A inhibition by anthocyanidins was 4,000-fold weaker than that achieved by clorgyline and MAO B inhibition was 200-fold weaker compared to inhibition from *R*-(−)-deprenyl. Proanthocyanidins B1 and B2 displayed only minimal enzyme inhibitory effects. In view of anthocyanins' and proanthocyanidins' instability in the intestinal environment [24,56,118], the role of phenolic acid metabolites was also addressed for MAO A. To judge by the present *in vitro* data, however, their activity is negligible (IC_{50} values > 3.9 mM), which further emphasizes the need to examine the bioavailability of parent compounds.

Overall, compounds under study featured little specificity with regard to inhibition of a particular enzyme isoform. For MAO A and B, maximum inhibition was achieved by anthocyanidins reaching IC_{50} values of < 23 µM, followed by monoglycosides and diglucosides. Anthocyanins' sugar moiety thus proved predictive of test compounds' potential for inhibiting MAO, in analogy to findings on other glucosylated flavonoids [511], albeit to a lesser extent. For both enzyme isoforms, the number of sugar moieties predicted IC_{50} values of anthocyanidins and their glycosides (MAO A, $p < 0.0001$, $F = 29.79$, $R^2 = 0.82$ and MAO B, $p = 0.0004$, $F = 15.51$, $R^2 = 0.71$). When a Bonferroni correction for multiple testing was applied, differences remained significant between anthocyanidins and anthocyanidin-3,5-diglucosides and between anthocyanidin-3-glycosides and anthocyanidin-3,5-diglucosides ($p < 0.001$) with respect to both MAO isoenzymes. In contrast, proanthocyanidins were weak inhibitors of both monoamine oxidases, and phenolic acids poorly inhibited MAO A.

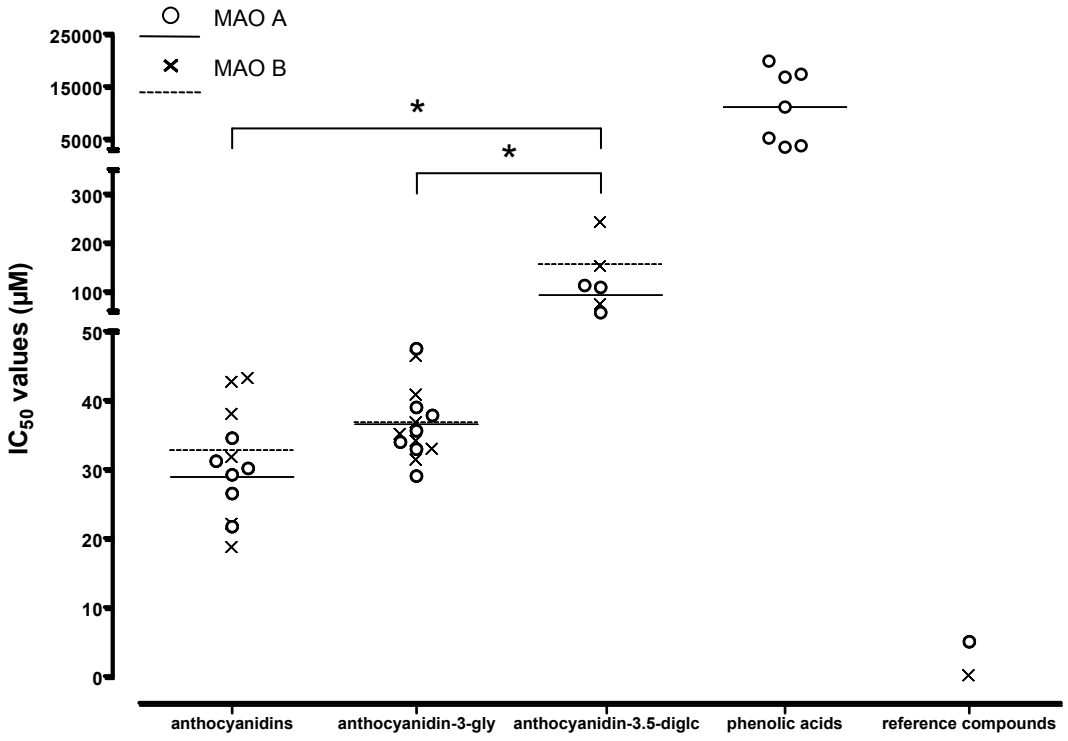

Figure 3-2 IC_{50} values (µM) of anthocyanidins, anthocyanidin-3-glycosides, anthocyanidin-3,5-diglucosides, phenolic acids, and the known inhibitors clorgyline (MAO A) and *R*-(−)-deprenyl (MAO B). Asterisks indicate significant differences between substance groups after Bonferroni correction at $p < 0.001$. Figure adapted from [513] and reprinted with permission from Elsevier.

Regarding qualitative effects on monoamine oxidases, for cyanidin-3-glucoside and its corresponding aglycone cyanidin, effects on MAO-catalyzed reactions were measured using three different inhibitor concentrations at increasing concentrations of substrate.

Kinetic data (table 3-4) and Lineweaver-Burk plots (LB plot) (figure 3-3) indicate competitive and mixed competitive and non-competitive modes of MAO A inhibition for cyanidin and cyanidin-3-glucoside, respectively. Whereas cyanidin showed a pronounced effect on the Michaelis-Menten constant (K_m) compared to the glucoside (increase by 426% versus 28%, respectively, at 80 µM), maximum enzyme velocity (V_{max}) was only marginally affected (decrease by 3.7% at 80 µM). In contrast, cyanidin-glucoside induced a major change in V_{max} (decrease by 48%). This suggests that cyanidin mediates inhibition of MAO A in a competitive manner, and the corresponding glucoside mediates inhibition in a mixed fashion. Dissociation constants of the enzyme-inhibitor complex, named K_i values, reached 20.4 µM and 47.4 µM for cyanidin and its glucoside, respectively. As for MAO B kinetics, cyanidin and cyanidin-3-glucoside acted as mixed competitive and non-competitive inhibitors. Unlike the differences seen in MAO A parameters, effects of the glucoside on MAO B K_m were comparable to those of cyanidin (increase by 23% and 27%, respectively, at 80 µM) as were effects on MAO B V_{max} (decrease by 59% and 57%, respectively). For cyanidin and cyanidin-glc K_i values of 58.6 µM and 61.1 µM, respectively, were obtained.

Table 3-4 Kinetic characteristics of cyanidin and cyandin-3-glucoside interactions with MAO A and MAO B. K_m and K_i values are expressed in µM. Mean values were formed from two separate experiments performed in duplicate.

	Control		Cyanidin		Cyanidin-3-glc	
	K_m	V_{max}	K_i	Type of inhibition	K_i	Type of inhibition
MAO A	25.6	2245	20.4	competitive	47.4	mixed
MAO B	5.2	242	58.6	mixed	61.1	mixed

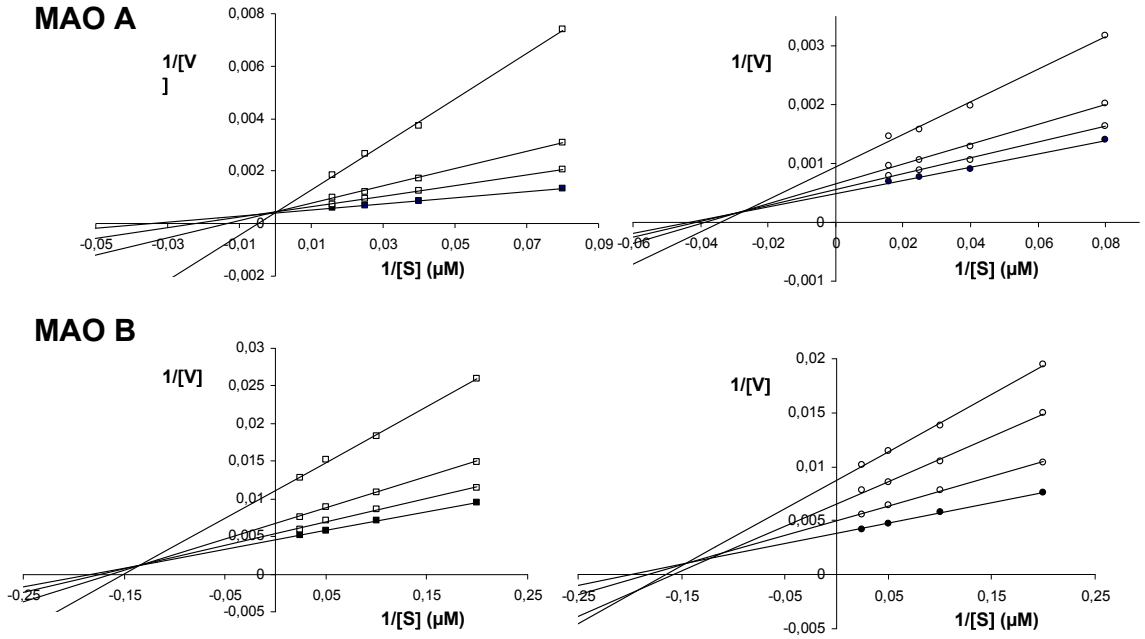

Figure 3-3 Inhibition-dissociation kinetics of cyanidin (left) (□) and cyanidin-3-glucoside (right) (○) for MAO A (top) and MAO B (bottom) relative to control values in the absence of test compounds (■,●). [V] = velocity of MAO-catalyzed reaction, [S] = substrate concentration. Reprinted from [513] with permission from Elsevier.

These data suggest that anthocyanins may hold promise in conditions where increased brain levels of biogenic amines are desirable [514], including deficiencies in norepinephrine and serotonin [515]. Specifically, MAO A inhibitory activity warrants further investigations of antidepressant and antianxiety functionalities. No effects were reported on anxiety levels of rats fed lyophilized berries in an elevated plus-maze paradigm [17]. More recent investigations using an anthocyanin extract, however, contradict this observation and argue in favor of anxiolytic properties [237]. Referring to available literature, antidepressant effects of anthocyanins have not been addressed. However, a study on older adults supplemented with wild blueberry juice for 12 weeks suggested reduced depressive symptoms and provided further corroboration of neurocognitive benefits associated with berry constituents [6].

In addition to their prominent role in the central nervous system, MAO enzymes may alter endogenous amine turnover in multiple peripheral tissues [516-518]. In the treatment of depression, nonselective and irreversible MAO inhibition has been associated with side effects outside the CNS [519]. For instance, when tyramine and other sympathicomimetic amines from fermented foods such as cheese enter the circulation and accumulate as a consequence of MAO inhibition in the intestine, sympathetic cardiovascular activity is potentiated by the release of norepinephrine [306]. Pending work on dissociation of anthocyanins from MAO A and B, food–drug interactions therefore cannot be excluded. As a significant number of subjects suffering

from depression consume dietary supplements [520], supplementation with large amounts of compounds from berry fruits may, in theory, predispose to adverse reactions in these individuals.

MAO B inhibition causes slowing of dopamine turnover in the mammalian brain [521] and limits the formation of hydrogen peroxide [522]. MAO B inhibitors may also reduce the secretion of neurotoxins and prevent generation of the toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), guarding against dopamine depletion in substantia nigra neurons [523]. Oxidative stress, depletion of antioxidants in the brain, and an increase in MAO B activity in reactive microglia are closely related risk factors in neurodegenerative disease [524,525]. Compounds that combine MAO B inhibitory with antiinflammatory and antioxidant properties may therefore offer significant advantages in arresting the underlying pathophysiological process [524,526]. Pending replication of the present findings in animal studies, anthocyanins may qualify as multifunctional agents by limiting the formation of free radicals, reducing the activation of environmental protoxins and by minimizing the generation of neurotoxic aldehydes.

In summary, MAO A and B inhibition by anthocyanins lends support to central nervous functionalities of diets rich in these polyphenols and opens new windows of opportunity in the prevention of neuronal pathologies. More research is invited to assess in detail the benefits to be derived from nutritional interventions with anthocyanins *in vivo*.

3.2.2 Effects on hypoxia inducible factors

Activation of a hypoxia signal transduction pathway presents an emerging target for neuroprotection associated with iron chelation. It is closely related to the expression of the transcriptional activator HIF and to increased transcription of genes mediating compensatory survival processes in response to oxidative stress. With respect to their modulatory effects on HIF-1α and HIF-2α expression, selected test substances were investigated in two neuroblastoma cell lines. As the presence of vicinal di-hydroxyl groups was shown to be a requirement for efficient iron-binding of phenolic compounds [527], anthocyanidins cyanidin and delphinidin plus their corresponding glucosides and possible phenolic metabolites were chosen as test substances. Procyanidin B2, anthocyanin-rich bilberry extract and the known HIF-1α inducers desferrioxamine and quercetin were also investigated.

3.2.2.1 Effects on SH-SY5Y viability

The influence of compounds under investigation on SH-SY5Y cell viability was assessed using the MTT test. When effects on MTT reduction were compared, a significant difference between test compounds was revealed ($p = 1.3 \cdot 10^{-5}$, $F = 16.74$, $df = 11$, $R^2 = 0.94$). After Dunnett correction for multiple comparisons, significant differences were observed for cyanidin-glc ($p = 0.0073$), cyanidin ($p = 0.0023$), procyanidin B2 ($p = 3.5 \cdot 10^{-5}$), protocatechuic acid ($p = 0.019$), gallic acid ($p = 0.018$) and DFO ($p = 0.024$) compared to control effects. As shown in figure 3-4, all test substances except gallic acid improved cell viability after a 4 h period of incubation.

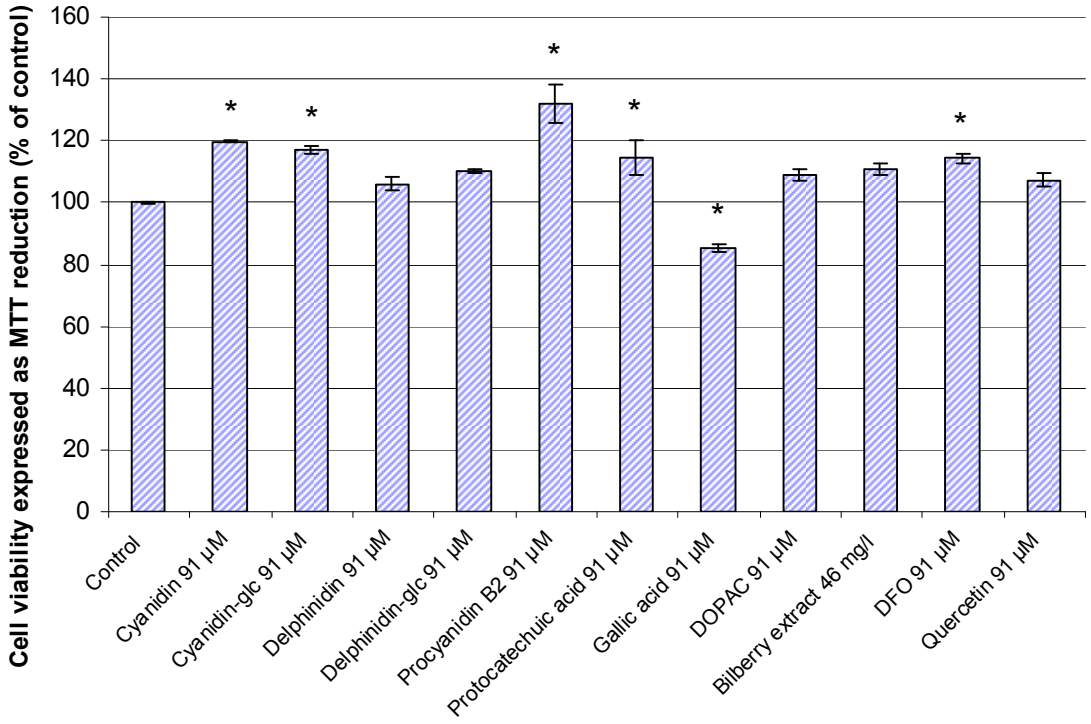

Figure 3-4 Effects of test substances on SH-SY5Y viability after 4 h incubation. Data points are means from two independent experiments with standard deviations shown as error bars. Asterisks indicate cell viabilities that differ from control viabilities after Dunnett correction for multiple testing at $p < 0.05$.

The MTT viability test implies that test substances with the exception of gallic acid do not exert cytotoxic effects on SH-SY5Y cells at concentrations of 91 µM during the 4 h incubation experiments.

3.2.2.2 Effects on HIF-α expression and cellular uptake

With regard to test substances' effects at 100 µM on HIF-1α protein expression after incubation for 4 h, significant differences compared to control were observed for both SH-SY5Y ($p = 3.3 \cdot 10^{-14}$, $F = 500.2$, $df = 11$, $R^2 = 0.998$) and IMR-32 cells ($p = 3.3 \cdot 10^{-11}$,

F = 156.8, df = 11, R^2 = 0.99) (figure 3-5). When Dunnett correction for multiple testing was applied, differences compared to control were significant for cyanidin ($p = 5.6 \cdot 10^{-4}$), delphinidin ($p = 3.7 \cdot 10^{-4}$), gallic acid ($p = 1.2 \cdot 10^{-4}$), bilberry extract (p = 0.0019), and the known HIF-1α inducers DFO and quercetin ($p = 8.8 \cdot 10^{-6}$) with respect to SH-SY5Y cells, and merely for DFO and quercetin ($p = 8.8 \cdot 10^{-6}$) in IMR-32 cells. With the exception of gallic acid, which reduced HIF-1α expression in SH-SY5Y cells, other substances with significant modulatory effects increased HIF-1α protein concentrations.

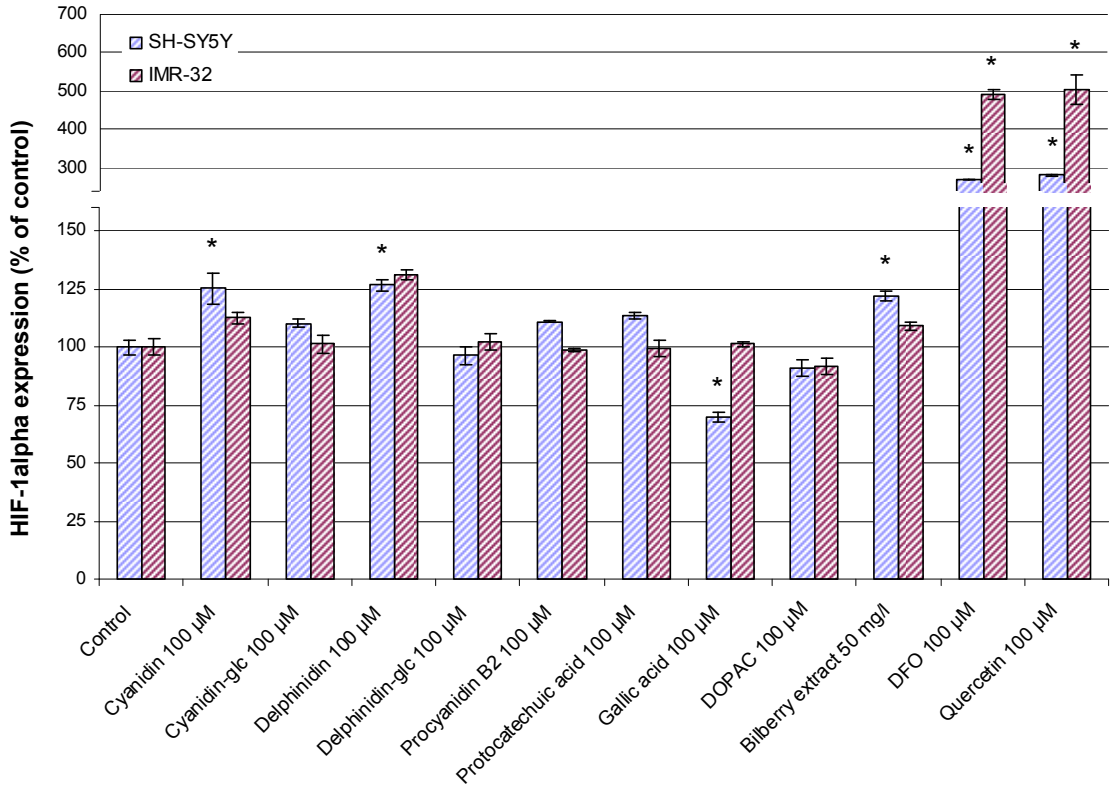

Figure 3-5 Effects of test substances on HIF-1α expression after 4 h of incubation. Data points are means from two independent incubation experiments with standard deviations shown as error bars. Asterisks indicate HIF-1α expression levels that differ from control levels after Dunnett correction for multiple testing at $p < 0.05$.

Likewise, test compounds' effects were significantly different from control for HIF-2α expression in both SH-SY5Y (p = 0.015, F = 3.83, df = 11, R^2 = 0.78) and IMR-32 cells (p = 0.0024, F = 5.88, df = 11, R^2 = 0.84) (figure 3-6). Following Dunnett correction for multiple testing, only bilberry extract exerted significantly different effects compared to controls in SH-SY5Y (p = 0.011) and IMR-32 cells (p = 0.023). In both cell lines, bilberry extract decreased HIF-2α protein concentrations.

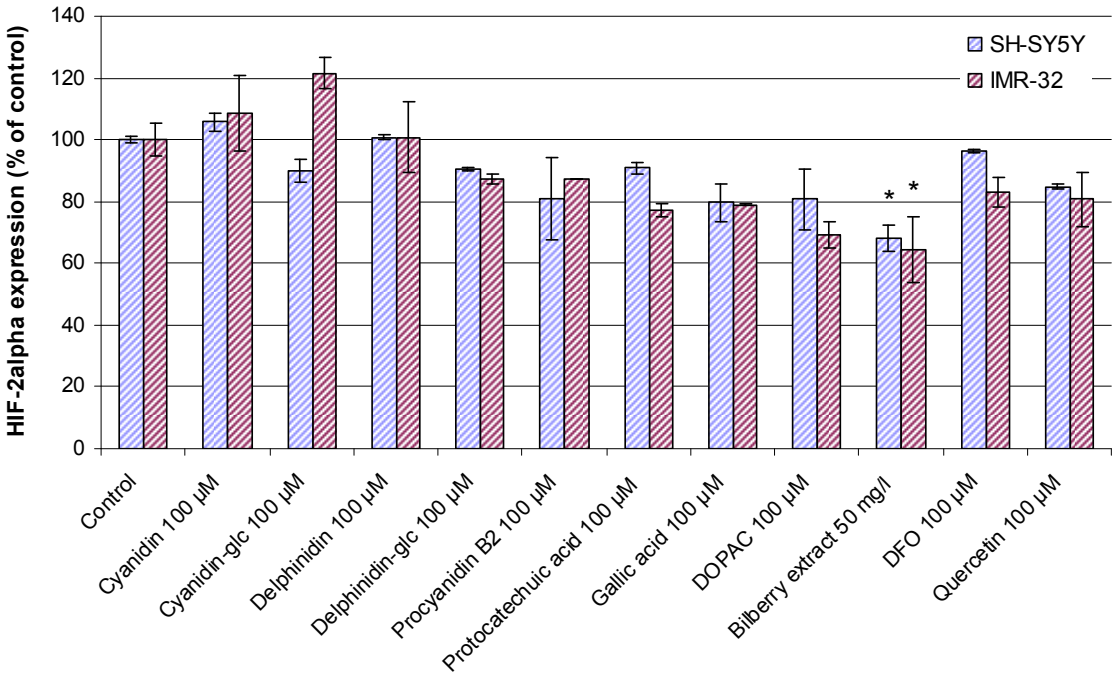

Figure 3-6 Effects of test substances on HIF-2α expression after 4 h of incubation. Data points are means from two independent incubation experiments with standard deviations shown as error bars. Asterisks indicate HIF-2α expression levels that differ from control levels after Dunnett correction for multiple testing at $p < 0.05$.

In this first round of substance screening experiments, the anthocyanidin delphinidin was the most promising compound regarding induction of HIF-1α expression. Since its potential to influence HIF-1α expression has not been previously addressed, delphinidin was selected for a second series of experiments. When effects of three delphinidin concentrations on HIF-1α protein expression were examined during 4 h of incubation, significant differences were observed in both SH-SY5Y ($p = 0.065$, $F = 5.56$, $df = 3$, $R^2 = 0.81$) and IMR-32 cells ($p = 0.00078$, $F = 63.89$, $df = 3$, $R^2 = 0.98$) compared to control (figure 3-7). For both cell lines a concentration-dependent increase of HIF-1α expression was found. Following Dunnett correction for multiple comparisons, HIF-1α expression was significantly different from control expression levels for delphinidin at 100 µM in SH-SY5Y cells ($p = 0.043$), and also for delphinidin at concentrations of 50 µM ($p = 0.0092$) and 100 µM ($p = 0.00060$) in IMR-32 cells.

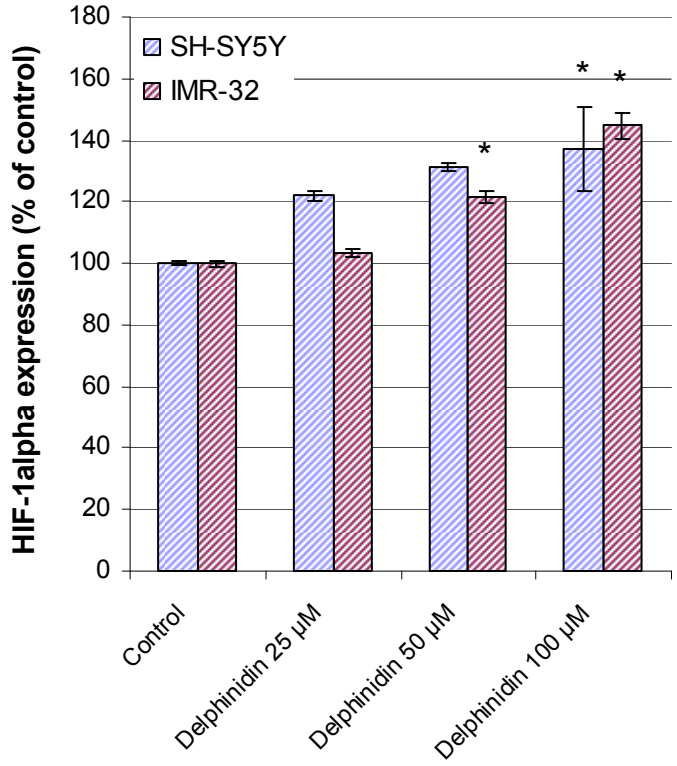

Figure 3-7 Concentration-dependent effects of delphinidin on HIF-1α expression after 4 h incubation. Data points are means from two independent incubation experiments with standard deviations shown as error bars. Asterisks indicate HIF-1α expression levels that differ from control levels after Dunnett correction for multiple testing at $p < 0.05$.

Since HIF-1α expression inducing effects were most conclusive for the highest delphinidin concentration tested, experiments were continued with delphinidin at 100 µM. Finally, a modulatory role of incubation time on HIF-1α intracellular protein levels was addressed. At all incubation times tested, increases in HIF-1α expression were significant compared to controls for SH-SY5Y cells (3 h: $p = 0.015$, $t = 8.02$, $df = 2$, $R^2 = 0.97$, 4 h: $p = 0.032$, $t = 5.44$, $df = 2$, $R^2 = 0.94$, 5 h: $p = 0.049$, $t = 4.36$, $df = 2$, $R^2 = 0.91$). In IMR-32 cells, pairwise t-tests revealed most pronounced and significant delphinidin effects at an incubation time of 4 h ($p = 0.0041$, $t = 15.62$, $df = 2$, $R^2 = 0.99$), followed by 3 h ($p = 0.012$, $t = 9.13$, $df = 2$, $R^2 = 0.98$).

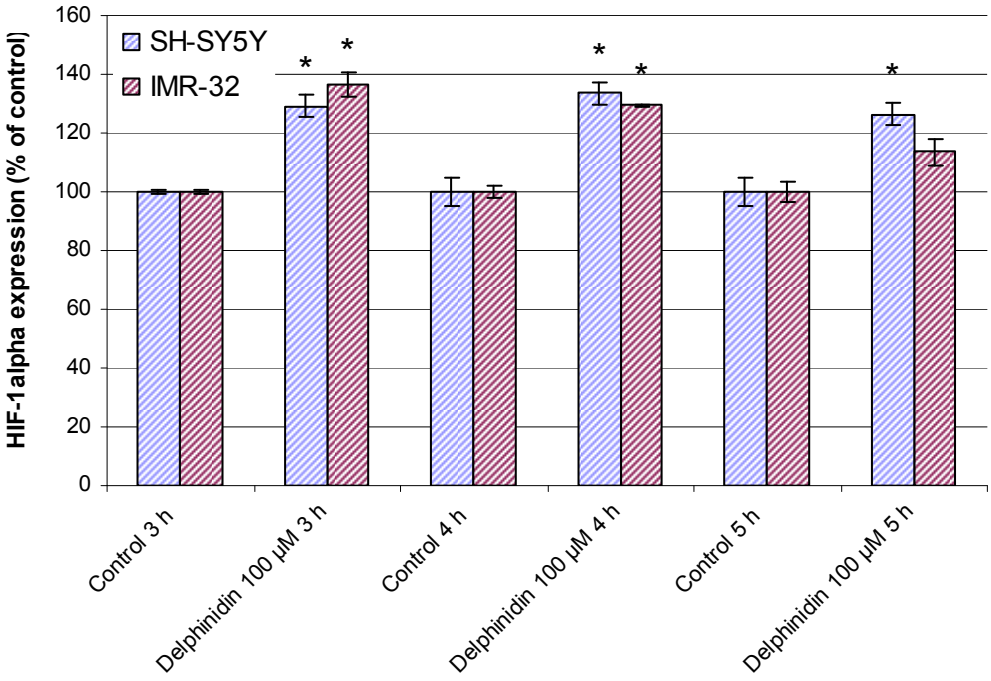

Figure 3-8 Incubation time-dependent effects of delphinidin on HIF-1α expression. Data points are means from two independent incubation experiments with standard deviations shown as error bars. Asterisks indicate HIF-1α expression levels that differ from the corresponding controls at $p < 0.05$, determined by pairwise t-tests.

As delphinidin turned out as potent modulator of HIF-1α expression in contrast to its glucoside, the question arose whether there may be differences in cellular uptake between those compounds and the known HIF-1α inducer quercetin.

Employing HPLC-VIS analyses, quercetin was identified and quantified in both SH-SY5Y and IMR-32 cell lysates following incubations at 100 µM for 4 h. Confirmation of the compound's identity was successfully conducted for both cell lines by means of HPLC-MS/MS, detecting the parent ion m/z 301.0 and the product ion m/z 151.2 in the single reaction monitoring (SRM) modus. While quercetin concentration amounted to 3.00 nmol/mg protein for SH-SY5Y cells, the IMR-32 sample yielded 4.52 nmol/mg protein.

With respect to delphinidin-glc, inconsistent results were obtained for the two cell lines. While no delphinidin-glc or other compounds were detected using HPLC-VIS in IMR-32 cells, the anthocyanin could be identified and quantified in SH-SY5Y cells (0.82 nmol/mg protein). However, substance identity could not be confirmed using HPLC-MS/MS since the concentration was below the detection limit as verified with a similarly concentrated delphinidin-glc standard. In addition, two unknown peaks appeared in the chromatogram with retention times of 27 and 43 min. Concerning identification of these unknown peaks, however, the sample amount was not sufficient to perform further HPLC-MS/MS analyses.

The anthocyanidin delphinidin was recovered from neither cell line. Again, unknown peaks with retention times of 27 min were found in lysates of both cell lines applying HPLC-VIS. From HPLC-DAD-MS analysis of the IMR-32 sample, generating a total ion chromatogram, predominantly an m/z of 204 emerged at the corresponding retention time of the unknown peak. However, delphinidin uptake cannot be entirely excluded in view of the low signal intensity and pending further HPLC-MS/MS experiments.

Finally, focus was specifically laid on identifying the previously described metabolites phloroglucinol and gallic acid [528] in delphinidin and delphinidin-glc samples of both cell lines, performing HPLC-MS/MS. While phloroglucinol was found in neither sample, gallic acid was identified in IMR-32 cells incubated with delphinidin-glc (figure 3-9). The presence of gallic acid in SH-SY5Y cells incubated with delphinidin is speculative since the compound concentration ranged around the limit of detection.

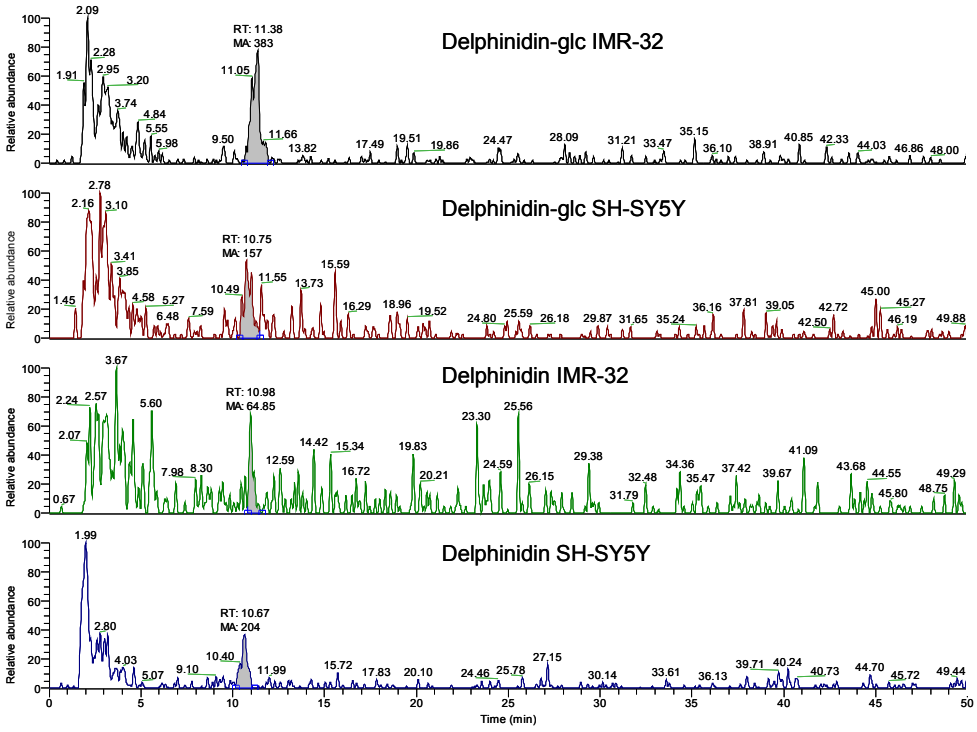

Figure 3-9 HPLC-MS/MS chromatograms measuring the ion transition of m/z 169.0 to m/z 125.1 characteristic for gallic acid. Areas under peaks at the retention time of gallic acid (10.5 min) are colored grey.

Findings on quercetin in cellular extracts upon incubations imply that the native flavonol may function as intracellular iron chelator and thus exert effects on HIF-1α expression.

Since anthocyanidins were found to induce HIF-1α expression, in contrast to anthocyanins, HIF induction may depend on the capability of test substances to penetrate the IMR-32 and SH-SY5Y cell membrane by passive diffusion or active transport before binding of intracellular iron. Since lipophilicity is directly linked to the

absence of hydrophilic sugar moieties, passive uptake of anthocyanidins may be more efficient and faster compared to uptake of their glucosides, leading to higher intracellular concentrations of anthocyanidins, and potential metabolites available for iron chelation.

The absence of delphinidin in lysates of both cell lines was not surprising, since aglycones are known to rapidly degrade at assay conditions during the incubation time of 4 h [55,63]. Therefore, delphinidin metabolites formed in the course of the incubation may mediate observed effects on HIF-1α expression. However, it cannot be determined whether delphinidin was degraded extracellularly followed by cellular absorption of HIF-1α active metabolites, or rather absorbed as intact molecule prior to intracellular degradation to active compounds. Identification of gallic acid in SH-SY5Y cells upon incubation with delphinidin suggests that this phenolic acid may be an HIF-1α modulatory metabolite. However, this seems unlikely given the presence of the phenolic acid in the IMR-32 lysate incubated with delphinidin-glc, which exerted no effect on HIF-1α expression. Therefore, delphinidin metabolites with modulatory effects on HIF-1α expression still need to be identified.

Although delphinidin-glc did not exert modulatory effects on HIF-1α expression, in SH-SY5Y cells this may not be due to prevention of anthocyanin uptake, as it may be possible in IMR-32 cells. This implies that in SH-S5Y cells, delphinidin-glc and extra- and intracellularly formed metabolites were ineffective at modulating HIF-1α expression at the concentrations they achieved in cells. Assuming that besides delphinidin aglycone its glucoside also became degraded to a great extent during the 4 h incubation period, it is surprising that no effect on HIF-1α expression was observed in samples incubated with the anthocyanin. One explanation may be the generation of different metabolites from the anthocyanin and its aglycone.

Various flavonoids have been identified as inducers of HIF-1α in various cell lines, including the flavones baicalein [529] and luteolin [530], the tea catechins EGCG [531] and ECG [532] and the flavonols galangin [533], fisetin [530] and quercetin [533-535]. HIF-1α inducing properties of quercetin at normoxic conditions are corroborated by the present results. Of above mentioned flavonoids, baicalein [529], quercetin [534,535], and ECG [532] also activated the angiogenic HIF downstream gene VEGF. As for HIF-2α, induction of expression was reported for quercetin and galangin [533]. In line with these findings on other flavonoids, anthocyanidins cyanidin and delphinidin or more likely their unknown metabolites induced HIF-1α expression in experiments conducted during the present work.

Anthocyanidins or emerging metabolites may be able to induce HIF-1α owing to their iron chelating properties [328]. Since iron constitutes a cofactor for prolyl-4-hydroxylase

activity, these phenolic substances may prevent HIF-1α hydroxylation and degradation. Then, HIF-1α subunits can dimerize with HIF-1β subunits and induce HIF-1-responsive genes. This iron-dependent mechanism has been substantiated by findings on the tea catechin ECG, demonstrating that induction of HIF-1α may be blocked by the addition of iron ions [532].

In consequence, tested anthocyanidins may exert beneficial effects on human health by transcriptional activation of downstream neuroprotective genes. As HIF-1α is thought to be one of the most crucial signalling molecules in tissue responses to hypoxia, regulating many downstream genes that are important in promoting cell survival such as EPO [344,536], glucose transporters [537], and vascular endothelial growth factor [538], anthocyanidins cyanidin and delphinidin and potential metabolites may protect neurons from ischemic damage as well as from other diseases associated with oxidative stress [344]. Therefore, HIF prolyl hydroxylase inhibition may, in addition to inhibition of Fenton chemistry, be a further mechanism by which iron chelators such as anthocyanidins and their phenolic metabolites may meditate protection from hypoxic injury and oxidative stress.

The well established role of prolyl hydroxylases in the scheme of HIF regulated gene transcription presents researchers a distinct therapeutic target to combat oxidative stress. The strategy of activating HIF by small molecule “drugs” such as anthocyanidins and potential degradation products has an advantage over prior “antioxidants”: Single substances may selectively target a single molecule such as PHD, mediating activation of more than seventy genes and thereby providing adaptation to ischemia and oxidative stress [342,345,352].

Nevertheless, further investigations are warranted to elucidate if anthocyanidins’ or their metabolites’ iron chelating properties can be associated with observed HIF-1α inducing effects and if HIF-1α induction by these substances has consequences for the expression of neuroprotective downstream genes, including EPO and VEGF. In this context, some flavonoidic compounds were demonstrated to stabilize HIF-1α, but at the same time to impair its nuclear accumulation and consequently HIF-1 transcriptional activity [530].

As opposed to these HIF-1α inducing results, the flavones apigenin [539-541] chrysin [542], the tea catechin EGCG [543] and grape seed extract [544] inhibited HIF-1α expression at low micromolar concentrations. In contrast to HIF-1α-inducing effects observed in colon epithelial cell lines in a normoxic environment [534], the flavonol quercetin suppressed HIF-1α accumulation during hypoxia in various cancer cell lines [545]. Flavonoids were also reported to downregulate the expression of the HIF downstream gene VEGF. In rats, chronic intake of red wine powder prevented the

stimulatory effect of angiotensin II on the ischemia-induced neovascularization and the accompanying augmented expression of proangiogenic factors including HIF-2α, VEGF and endothelial nitric-oxide synthase (eNOS) [546].

Findings on HIF inhibitory effects as shown for gallic acid with respect to HIF-1α and for bilberry extract, procyanidin B2 and phenolic acids with respect to HIF-2α add new insight into the potential mechanisms of substances' and their metabolites' anticancer activities. Since HIF-1 is overexpressed in many human cancers [547] and the levels of its activity in cells was shown to correlate with tumorigenicity and angiogenesis [548], these polyphenols may be effective in attenuating tumor cell proliferation and may reveal potencies in cancer treatment. In line with these findings, gallic acid exerted antiangiogenic effects in a xenograft mouse model [549]. In addition, these HIF downregulating compounds may prove protective in conditions of diabetic retinopathy, as VEGF expression seems to be implicated in the abnormal vascularization observed in pathological conditions including diabetic retinopathy [550,551].

In summary, protective effects of anthocyanidins or presumably of possible metabolites may benefit from substances' properties to antagonize oxidative stress in the brain at multiple levels. Besides their activities as direct radical scavengers, iron chelating properties may inhibit hydroxyl radical formation via Fenton chemistry and may induce expression of HIF and downstream neuroprotective genes. Therefore anthocyanins may be recognized as novel multifunctional therapeutics that can prevent or delay neuronal death in the degenerating human brain. However, the brain therapeutic future of active berry constituents relies on whether their neuroprotective actions can be successfully translated into prospective human studies.

3.2.4 Effects on the proteasome

The proteasome counts among further targets multifunctional berry flavonoids may interact with to exert neuroprotective functions. In continuation of earlier research focusing on the inhibition of proteasome activity by grape extract [374], this experiment was conceived to examine 10 anthocyanins', seven anthocyanidins', and two proanthocyanidins' *in vitro* impact on the chymotrypsin-like (ChT-L) proteasome activity. Table 3-5 summarizes IC_{50} values of all flavonoids under study.

Table 3-5 IC_{50} values calculated for inhibition of proteasomal chymotrypsin-like activity in HL-60 cells by anthocyanidins, anthocyanins and procyanidins.

Test substances	IC_{50} in µM (95% confidence interval)
Kaempferidinidin	7.8 (7.1-8.4)
Pelargonidin	7.8 (7.2-8.4)
Peonidin	9.0 (8.1-10.0)
Cyanidin-3,5-diglc	11.0 (9.8-12.4)
Cyanidin-3-glc	12.6 (10.9-14.5)
Peonidin-3-glc	12.8 (11.5-14.2)
Delphinidin-3-glc	13.0 (11.9-14.2)
Cyanidin-3-gal	13.5 (11.8-15.6)
Malvidin-3,5-diglc	17.1 (15.7-18.6)
Cyanidin-3-rut	18.1 (15.9-20.6)
Cyanidin	18.4 (16.4-20.7)
Malvidin-3-gal	21.7 (19.6-23.9)
Malvidin-3-glc	23.2 (21.1-25.6)
Petunidin	23.7 (20.8-27.0)
Malvidin	32.0 (29.7-34.4)
Pelargonidin-3,5-diglc	32.3 (28.1-37.1)
Delphinidin	32.4 (29.4-35.8)
Procyanidin B1	> 100 (-)
Procyanidin B2	> 100 (-)

Considering anthocyanins and anthocyanidins investigated, IC_{50} values for inhibition of the proteasomal chymotrypsin-like activity differed by a factor of 4. Anthocyanins and their aglycones inhibited proteasome activity in a concentration-dependent manner and achieved IC_{50} values between 7.8 and 32.4 µM. Overall, the anthocyanidins kaempferidinidin, pelargonidin and peonidin acted as powerful inhibitors with IC_{50} values of 7.8, 7.8, and 9.0 µM, respectively. The least potent inhibitors were identified as malvidin, pelargonidin-3,5-diglucoside and delphinidin, featuring IC_{50} values above 30 µM.

These data imply that the previously reported proteasome inhibitory activity of grape extract [374] was at least in part mediated by anthocyanins, and also suggest that anthocyanin concentration may aid in predicting the activity of other fruit and vegetable extracts. Anthocyanins and their aglycones achieved IC_{50} values comparable to those of other flavonoids. For the flavone apigenin, the flavonols quercetin, kaempferol and myricetin [375], and the tea flavanol EGCG [376], IC_{50} values ranged from 1 to 18 µM. Although the absence of sugar moieties from these flavonoids would appear to suggest a higher inhibitory potency of aglycones, this cannot be confirmed by the present

results. When compounds are grouped according to the presence or the absence of a sugar moiety, no trend is seen with regard to proteasome inhibition. Mean IC_{50} values of anthocyanins (IC_{50} = 17.5 +/– 6.6 µM SD) and anthocyanidins (IC_{50} = 18.7 +/– 11.0 µM SD) show only a marginal difference (p = 0.78, t = 0.28, df = 15). This finding is further illustrated by the inhibitory profiles of the two anthocyanins pelargonidin-3,5-diglucoside and delphinidin-3-glucoside and their corresponding aglycones (figure 3-10). For the first pair of compounds, pelargonidin, the aglycone, acted as a powerful proteasome inhibitor (IC_{50} = 7.8 µM), whereas pelargonidin-3,5-diglucoside showed more moderate inhibition (IC_{50} = 32.3 µM). For the second pair, however, inhibition by the aglycone delphinidin was less pronounced (IC_{50} = 32.4 µM) than that achieved by the corresponding delphinidin-3-glucoside (IC_{50} = 13.0 µM). For the procyanidin dimers B1 and B2, no inhibitory effects on proteasome activity were observed at any of the concentrations tested.

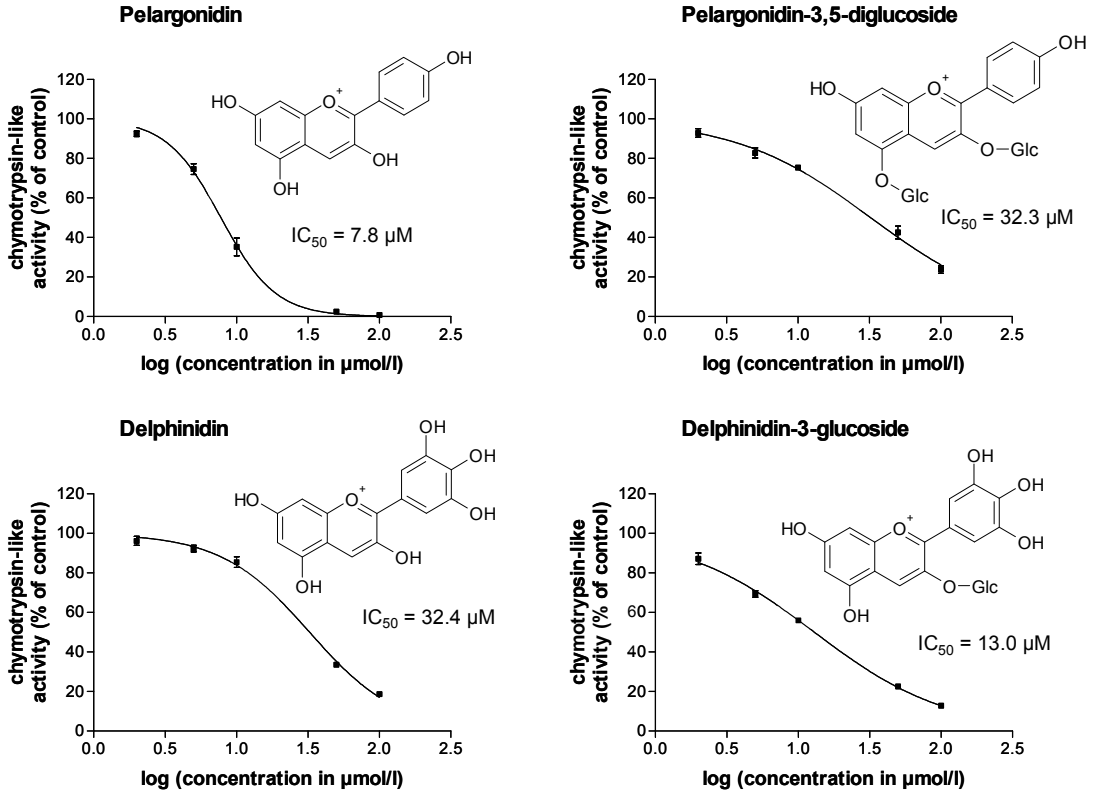

Figure 3-10 Inhibition of the chymotrypsin-like proteasome activity by pelargonidin, delphinidin, and the respective glucosides, pelargonidin-3,5-diglucoside and delphinidin-3-glucoside: Data points are means with standard deviations shown as vertical error bars of three independent experiments performed in triplicate. Reprinted from [552], with permission from Elsevier.

Regarding the substitution-pattern of anthocyanins' B-ring, mixed effects were noted on proteasome inhibition. Since the majority of effective inhibitors carries only one or two hydroxyl or methoxyl substituents on their B-ring, as opposed to three substituents in

most of the remaining compounds, inhibitory potency may be sensitive to this structural feature.

For the dimeric procyanidins B1 and B2, the absence of effects in the present study does not imply that these substances cannot inhibit the proteasome. The chymotrypsin-like activity is generally considered the rate-limiting activity in protein breakdown [553], but dimeric procyanidins may inhibit other catalytically active sites of the proteasome, e.g. the “trypsin-like” or the “postglutamyl hydrolyzing” activity [372], or may exhibit proteolytical activities independent of the proteasome [554].

It should also be noted that cells were permeabilized to minimize any impact of transport parameters on measurements of proteasome inhibition. Only few data are currently available on anthocyanins’ cellular uptake, intestinal absorption and transport across the BBB, which limits extrapolations to *in vivo* effects.

Clinical applications of proteasome inhibition have focused on tumor suppression [555], and a growing body of evidence supports additional roles in neuroprotection. Among the downstream effects of proteasome inhibition already identified counts the upregulation of heat shock proteins (HSP) [556] and of enzymes involved in antioxidant defense [557], plus the suppression of the proinflammatory immune response [558]. Specifically, the expression levels of HSP22 and HSP70 were reported to be increased following proteasome inhibition [559], an effect believed to prevent protein misfolding and the formation of protein aggregates. These cytoprotective qualities allow cells to survive under otherwise lethal conditions [560] and may slow the course of neurodegenerative disorders by refolding denatured proteins, as shown in models of Huntington’s disease [561].

Furthermore, multiple antioxidant enzymes have been shown to be highly expressed following proteasome inhibition, including superoxide dismutase I [557], thioredoxin reductase I, peroxiredoxins I and VI, and metallothioneins I and II [559]. Moreover, it has been demonstrated that proteasomal inhibition affords cytoprotection against oxidative stress by inducing glutathione synthesis in animal models of Parkinson’s disease [361]. Of all organs, the brain is most susceptible to oxidative damage due to its high oxygen demand [562]. Here, by protecting against oxidative and nitrosative stress [563,564], anthocyanins and anthocyanidins likely limit damage of brain cells at the protein, membrane lipid, and DNA levels.

Inhibitors of the proteasome may also affect the immune response by repressing antigen presentation on major histocompatibility complex (MHC) class I receptors [565], by suppressing cytokine secretion, cell-cell-interactions, migration and chemotaxis of lymphocytes, and by inducing apoptosis in activated T-cells. Downregulation of cytokine secretion [566] and cell adhesion molecule expression

[567] occurs via NF-κB inactivation mediated by the proteasome [362]. It is suggested that activation of the NF-κB pathway may play a role in a number of acute and chronic diseases with an inflammatory component, such as atherosclerosis, asthma, rheumatoid arthritis, inflammatory bowel disease [568], and neurodegenerative disorders such as cerebrovascular disease, Parkinson's disease and Alzheimer's disease. In line with this assumption, rats fed a blueberry diet showed a decline in age-related cognitive deficits and a reduction in NF-κB expression compared to non-supplemented controls [233,274].

Finally, it appears that rapidly dividing cells are more sensitive to proapoptotic effects of proteasome inhibitors than differentiated or non-proliferating cells [569], which can be advantageous in tumor therapy.

In summary, dietary supplementation with berry constituents may serve to prevent a number of common diseases by interfering with the proteasome pathway. Inhibition of the proteasome by anthocyanins and anthocyanidins adds to our understanding of cellular effectors that may control antiinflammatory, immunomodulatory and neuroprotective activities of these substances. Further research is invited to address effects of possible anthocyanin metabolites and downstream mechanisms upon proteasome inhibition.

3.2.4 Effects on Phospholipase A_2

Due to associations between PLA_2 activity and oxidative stress and inflammation, berry constituents may also confer neuroprotection by interacting with these enzymes. Thus berry constituents' *in vitro* impact on PLA_2-V activity was assessed by screening nine anthocyanins and six anthocyanidins, procyanidin B2, protocatechuic acid and catechin. Of the compounds examined, anthocyanidins exhibited the best inhibitory effects on PLA_2 in a first round of experiments providing dose-response relationships, whereas inhibitory properties of anthocyanins, in contrast, were less pronounced. For anthocyanins, corresponding aglycones and procyanidin B2, inhibition could not be quantified as absorption interfered with the photometric assay at millimolar concentrations and for some compounds dose-response curves implied incomplete $sPLA_2$-V inhibition. Catechin, the flavan-3-ol analog of cyanidin, and protocatechuic acid, a potential cyanidin metabolite, reached 50% inhibition at concentrations of 2.5 mM and 3.3 mM, respectively. Further investigations of enzyme kinetics were therefore restricted to the aglycones cyanidin, malvidin, peonidin, petunidin, delphinidin and pelargonidin.

From LB plots K_m (0.3 mM) and V_{max} (14 µmol/min/ml) were determined, and kinetic parameters are summarized in table 3-6. With regard to the mode of interaction with PLA_2, only the reference compound thioetheramide phosphatidylcholine (K_i = 0.59 µM) exhibited complete competitive inhibition. For malvidin (K_i = 6.4 µM), a hyperbolic slope LB plot versus inhibitor concentration [I] replot was obtained, indicating partial competitive PLA_2 inhibition at α = 1.8 and assuming β = 1. LB plots for pelargonidin (K_i = 325 µM) and delphinidin (K_i = 18 µM) meet criteria for mixed competitive and non-competitive PLA_2 inhibition. For both compounds, linearity of the LB plot slope versus [I] replot confirms complete inhibition at α values of 14.8 and 1.6 for delphinidin and pelargonidin, respectively. Petunidin (K_i = 14 µM), peonidin (K_i = 10 µM) and cyanidin (K_i = 2.1 µM) can also be identified as mixed competitive and non-competitive inhibitors from LB plots. However, their LB plot slope versus [I] replots indicate a partial (hyperbolic) type of inhibition. For these flavonoids, the ternary complex rate coefficients α and β (figure 4-10) were calculated from the linear plots of 1/Δslope versus 1/[I] and 1/Δordinate intercept versus 1/[I] [570], yielding values of 1.6, 1.6 and 2.9 (α) and 0.62, 0.79 and 0.7 (β) for petunidin, peonidin and cyanidin, respectively.

Table 3-6 Kinetic parameters of PLA_2 inhibition by anthocyanidins. Thioetheramide phosphatidylcholine (PC) served as a reference inhibitor. Table adapted from [571] and used with kind permission from Springer Science + Business Media (http://www.springerlink.com/content/e673663778663761).

Test substance	type of inhibition	Ki (µM)	α	β
Thioetheramide-PC	competitive	0.59		
Cyanidin	partial mixed	2.1	2.9	0.70
Malvidin	partial competitive	6.4	1.8	
Peonidin	partial mixed	10	1.6	0.79
Petunidin	partial mixed	14	1.6	0.62
Delphinidin	mixed	18	14.8	
Pelargonidin	mixed	325	1.6	

These results demonstrate $sPLA_2$-V inhibition by anthocyanidins in the low micromolar range (K_i = 2.1–18 µM), with the exception of pelargonidin (K_i = 325 µM). For cyanidin, inhibition approached that of the reference $sPLA_2$ inhibitor, thioetheramide phosphatidylcholine, with K_i values differing by a factor of 4. Anthocyanidin-glycosides, in contrast, were weak PLA_2-V inhibitors for which K_i values could not be estimated as anthocyanins' absorption at higher concentrations interfered with the photometric assay. Thus, with the exception of pelargonidin, the aglycones of prevalent anthocyanins from food sources are potent PLA_2 inhibitors.

Other than anthocyanidins, a limited number of flavonoids have been tested for PLA_2 inhibitory activity. Among these, the flavonols quercetin, quercetagetin and kaempferol-3-*O*-galactoside, plus the flavone scutellarein inhibited PLA_2-II with IC_{50} values ranging from 2 to 18 µM [572,573]. For PLA_2-V, the flavonol derivate papyriflavonol A and the biflavonoids amentoflavone and ochnaflavone showed 50% inhibition at concentrations between 5 and 42 µM [574,575], but K_i values are lacking. Four of the six tested anthocyanidins exerted only partial inhibition of PLA_2, as has also been observed for PLA_2-I with the flavonol quercetin and its 3-rutinoside rutin [573,576].

With regard to structural features, similar K_i values for most anthocyanidins investigated argue against a major role of anthocyanidins' B-ring substitution pattern in predicting $sPLA_2$-V inhibitory potential. To judge by weak inhibitory activity of catechin, the flavan-3-ol analogon of cyanidin (IC_{50} = 2.5 mM), anthocyanidins' unsaturated C-ring or their electric charge may prove more informative. With respect to the type of PLA_2 inhibition, however, B-ring substitution patterns deserve further study.

As natural anthocyanins are reportedly unstable in the intestinal environment, the role of phenolic acid metabolites generated by intestinal microflora and metabolic degradation is of particular interest [24,56,70]. However, follow-up experiments conducted with protocatechuic acid, a potential cyanidin metabolite, elicited only very weak $sPLA_2$-V inhibition (IC_{50} = 3.2 mM). Bioavailability of individual parent compounds therefore requires further study prior to assuming *in vivo* inhibitory effects.

For those agents that exhibit *in vitro* activities in the low micromolar range, a number of possible CNS functionalities may be discussed. Recent studies implicate increased PLA_2 activity and PLA_2-generated mediators in the acute inflammatory response of the brain, e.g., to ischemia [386], in kainic acid-induced neurotoxicity [577], and in chronic pathologies associated with Alzheimer's disease, Parkinson's disease, multiple sclerosis [386], schizophrenia [578,579], and bipolar affective disorder [580]. PLA_2 cellular effects may manifest at multiple levels. Phospholipid breakdown may increase membrane permeability and, consequently, Ca^{2+} influx, lipolysis, and proteolysis [581]. Lysophospholipids, in turn, may exert detergent-like effects on neuronal membranes [582] and act as precursors of the platelet-activating factor (PAF), a strong mediator of the inflammatory process [382]. Free fatty acids released from phospholipids can alter mitochondrial polarization state [583], cause mitochondrial dysfunction and may trigger an uncontrolled arachidonic acid cascade, followed by synthesis of inflammatory mediators, production of ROS [581] and neurotoxic 4-hydroxynonenal [387]. Released arachidonic acid, finally, may alter membrane fluidity [584], inhibit glutamate uptake [585], and modulate activities of protein kinases [586].

Neuroinflammation, oxidative stress, and altered phospholipid metabolism are involved in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and multiple sclerosis, leading to neuronal loss via a complex sequence of events that comprise an upregulation of complement, cytokines, and acute phase reactants among other mediators [386,587-589]. In this context, there is growing support for strategies that prevent inflammatory reactions during neurodegeneration. Mixed results have been achieved by inhibiting selective pathways of eicosanoid production, i.e. LOX and COX pathways [382]. Control of arachidonic acid production currently holds promise in the treatment of phospholipid pathologies. A challenge in maintaining basal levels of arachidonic acid, lysophospholipids, and PAF, however, is posed by the multiplicity of PLA_2s, the interplay among downstream mediators and the recognition that many PLA_2 functionalities are also essential for normal cell function [590].

Moreover, with regard to the etiology of most disorders, it remains to be established whether phospholipid breakdown is present early in neurodegenerative disease or whether it is only an epiphenomenon of cell death [591]. Pending an improved understanding of cause and effect, the utility of candidate PLA_2 inhibitors in counteracting phospholipid degradation is difficult to predict by *in vitro* data.

Should PLA_2 inhibition occur at the concentrations achieved by dietary intake of anthocyanins, this may help explain certain fruits' role in lowering age-related neurodegenerative disease [252,258]. Although oxidative stress [592] and inflammatory reactions [254] both contribute to age-related pathologies, antioxidant activity alone does not explain the potency of berry constituents in protecting against neurodegeneration [10]. Anthocyanin effects on phospholipid metabolism may help explain such benefits as does inhibition of lipid peroxidation [277] and modulation of inflammatory mediators COX I and II [62]. Partial inhibition of PLA_2-V by most compounds under study may prove advantageous *in vivo* in that basal levels of phospholipid-derived mediators could be maintained for normal brain function.

Taken together, beneficial effects of fruit antioxidants on aging and neurodegeneration warrant investigations at multiple levels. The present findings on $sPLA_2$-V inhibition by anthocyanidins provide further evidence to rationalize antioxidative and antiinflammatory activities. More studies are invited to explore PLA_2 isoform specificity of these properties, and to define their behavioral correlates.

For interactions with brain enzymes and neuroprotective mechanisms including monoamine oxidases A and B, hypoxia inducible factor, proteasome and phospholipase A_2 to take effect, anthocyanidins and anthocyanins need to enter the

brain at micromolar concentrations. Existing data from human trials point to peak plasma concentrations in the nanomolar range [70,93,488]. Although anthocyanin brain bioavailability is not sufficiently elucidated to date and raises several questions, results from animal studies would appear to rationalize interactions with brain monoamine oxidases A and B, hypoxia inducible factor, proteasome and phospholipase A_2 *in vivo*, as these agents rapidly cross the blood-brain barrier [80,83,112-114]. Favorable changes in central nervous parameters of oxidative stress following administration of anthocyanins further corroborate this hypothesis [16] but detailed tissue quantitations of anthocyanins and anthocyanidins have yet to be performed.

Moreover, available estimates on anthocyanin bioavailability may be considered conservative in that analytical challenges are posed by anthocyanin accumulation in tissues as shown for rats [82], by metabolic transformation to molecular structures that are not routinely detected, and by protein binding [115,489,490].

Assuming anthocyanins' low stability under assay condition at neutral pH values and in the human body, reflected by low recovery rates from human plasma and urine and different animal tissues, native anthocyanins, aglycones and phenolic compounds tested in the present work may be different from compounds primarily generated during *in vitro* assays and in the human body upon oral uptake. In consequence, anthocyanins and anthocyanidins may not exclusively be accountable for positive effects observed *in vitro* and *in vivo*. Therefore, extrapolations of anthocyanins' and anthocyanidins' *in vitro* neuroprotective effects to the *in vivo* situation do advocate caution. Although a first approach was undertaken by investigating effects of phenolic acids, as they are known metabolites, further research is invited to address identification of additional *in vivo* and *in vitro* metabolites.

Moreover, substance uptake into cells may be different for various cells in the human body and for cell lines used *in vitro*, as exemplified upon incubations of two neuroblastoma cell lines with delphinidin-glc. This also needs to be kept in mind for extrapolation of compounds' *in vitro* cellular effects to other systems such as the human body.

In vitro studies performed within the current work have the advantage over i*n vivo* studies that pure anthocyanins can be tested in contrast to complex matrices usually applied *in vivo*. For instance, caution should be advised, when health benefits observed during in vivo studies upon administration of grape seed extracts are ascribed to proanthocyanidins constituting up to 75-90% of GSPE [209,419,593]. In contrast, *in vitro* results of the present work can be exclusively attributed to the single compounds under investigation. On the other hand, when extrapolating *in vitro* neuroprotective

effects to humans, food matrices' effects should not be underestimated. These may include synergistic and quenching effects caused by other food ingredients.

3.3 Effects on xenobiotic metabolism and safety

3.3.1 Effects on cytochrome P450 3A4

It has been shown that anthocyanins themselves are not metabolized by cytochrome P450 enzymes [57], but the rapidly growing interest in dietary anthocyanins calls for a more thorough understanding of their cellular targets and possible hazards posed by interference with the metabolism of common drugs. To extend earlier studies of grapefruit and polyphenolic green tea, wine, and apple constituents and to further elucidate substances' potential to interact with xenobiotics metabolism, 10 anthocyanins', six anthocyanidins' and seven phenolic acids' modulatory effects on cytochrome P450 3A4 activity were investigated, along with effects of procyanidins B1 and B2.

Test compounds inhibited CYP3A4 activity in a concentration-dependent manner and gave distinct profiles for the substance groups under study. An overview on IC_{50} values is given in table 3-7. For anthocyanidins, IC_{50} values ranged from 12 to 47 µM (mean 22 +/– 13 µM SD), for anthocyanidin-monoglycosides from 74 to 105 µM (mean 86 +/– 11 µM SD), for anthocyanidin-diglucosides from 166 to 249 µM (mean 214 +/– 41 µM SD), for phenolic acids from 472 up to 7,842 µM (mean 5,546 +/– 3,130 µM SD) and for procyanidins B2 and B1 540 and 780 µM (mean 660 +/– 170 µM SD). Thus, phenolic acids acted as the weakest inhibitors, followed by procyanidins, anthocyanins and anthocyanidins. Ketoconazole, a widely used inhibitor that had been chosen for a reference, reached an IC_{50} value of 18.4 nM.

Table 3-7 IC_{50} values calculated for inhibition of CYP3A4 activity by anthocyanidins, anthocyanins, procyanidins, phenolic acids and ketoconazole.

Test compound	IC_{50} in µM (95% confidence interval)
Pelargonidin	12 (12-13)
Malvidin	14 (13-16)
Peonidin	16 (15-18)
Cyanidin	21 (19-22)
Petunidin	23 (22-25)
Delphinidin	47 (43-51)
Delphinidin-3-glc	74 (68-81)

Peonidin-3-glc	79	(74-85)
Malvidin-3-glc	80	(73-88)
Cyanidin-3-gal	80	(75-86)
Malvidin-3-gal	84	(77-92)
Cyanidin-3-rut	96	(87-106)
Cyanidin-3-glc	105	(96-115)
Cyanidin-3,5-diglc	168	(154-183)
Malvidin-3,5-diglc	226	(203-250)
Pelargonidin-3,5-diglc	249	(206-301)
Procyanidin B2	540	(472-618)
Procyanidin B1	780	(628-968)
Vanillic acid	472	(441-506)
Syringic acid	1,622	(1,442-1,824)
3-Hydroxyphenylacetic acid	6,558	(6,364-6,759)
3-(4-Hydroxyphenyl)propionic acid	6,841	(6,528-7,170)
Protocatechuic acid	7,666	(7,435-7,904)
4-Hydroxybenzoic acid	7,821	(7,633-8,014)
4-Hydroxyphenylacetic acid	7,842	(7,520-8,177)
Ketoconazole	18 nM	(16-21 nM)

Of the polyphenolic compounds previously investigated, naringenin, EGCG, quercetin, ECG, curcumin and resveratrol exhibited IC_{50} values of approximately 87 µM [594], 40 µM [595], 38 µM [596], 20 µM [595], 16 µM [597] and 4 µM [598], respectively. Furanocoumarins from grapefruit juice, however, set an activity benchmark in the nanomolar range. Specifically, bergamottin, 6',7'-dihydroxybergamottin and the dimerics GF-I-1 (4-[[6-hydroxy-7-[[1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-*g*][1]benzopyran-7-one) and GF-I-4 (4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7H-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-*g*][1]benzopyran-7-one) exhibited IC_{50} values as low as 1,000 nM, 250 nM, 3 nM and 3 nM for CYP3A4, respectively [438].

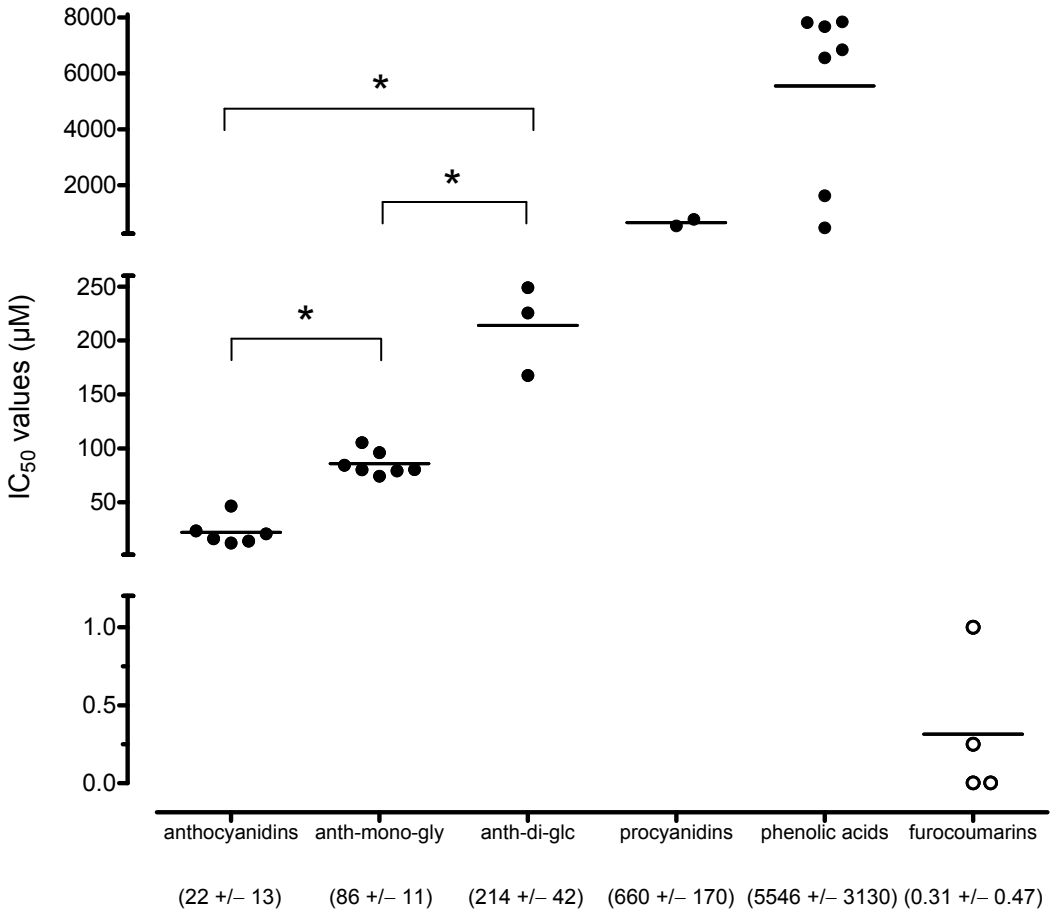

Figure 3-11 IC_{50} values of anthocyanidins, anthocyanidin-3-glycosides (anth-mono-gly), anthocyanidin-3,5-diglucosides (anth-diglc), anthocyanidins, procyanidins and phenolic acids compared to IC_{50} values of furanocoumarins [438]. Mean IC_{50} values +/- SD (µM) are given in brackets. Asterisks indicate differences between substance groups after Bonferroni correction at $p < 0.001$. Figure adapted from [599], copyright Wiley-VCH Verlag GmbH & Co. KGaA, reproduced with permission.

In comparison, CYP3A4 inhibitory effects of anthocyanins, their aglycones, dimeric procyanidins and phenolic acids are weaker by several orders of magnitude (figure 3-11). Relative to ketoconazole, an antifungal and known inhibitor, the flavonoids investigated here feature a 1,000 to 10,000-fold lower potential for interactions (> 100,000-fold lower for phenolic acids). Thus, these results suggest that anthocyanins, procyanidins and phenolic acids pose only a limited risk of food-drug interactions mediated by CYP3A4 as compared to other grapefruit, wine, green tea and apple juice constituents. Therefore, anthocyanins' and their metabolites' contribution to CYP3A4 inhibitory activity in wine and berry juices may only be minor [446,448,600].

When the number of sugar moieties per compound is used to predict IC_{50} values of anthocyanins, a significant impact is noted on CYP3A4 inhibitory functionality ($p < 0.0001$, $F = 94.69$, $R^2 = 0.94$). When a Bonferroni correction for multiple testing is applied to one-way analysis of variance (ANOVA) for differences between compounds

with an unequal number of sugar moieties, p values remain significant at the level of 0.001. However, substances' B-ring substitution pattern is not predictive of their CYP3A4 inhibitory properties. Anthocyanidins' glycosylation may play a dual role with regard to *in vivo* effects. In addition to impacting the absorption of compounds [91,601], sugar moieties have been shown to enhance cytochrome inhibitory activity of some flavonoids [602]. In contrast, in the present investigations, the number of sugar moieties predicted a decline in anthocyanidins' effects on CYP3A4, which underscores the need for more detailed data on structure-activity relationships.

Before an extrapolation of *in vitro* data to *in vivo* effects can be attempted, additional parameters must be taken into account. For instance, CYP3A4 may be more susceptible to inhibition by food constituents than other mainly hepatic cytochrome P450 isoforms. As high levels of CYP3A4 expression in the intestine allow interactions during the digestive process [603], substances need not cross the intestinal barrier in order to interfere with metabolism. Even when oral bioavailability is low, compounds may act as potent inhibitors, provided that they are able to penetrate epithelial cells in the intestine. Therefore, anthocyanins' and anthocyanidins' impact on metabolic drug processing by CYP3A4 is also dependent on transport into intestinal epithelium cells.

Finally, further characterization of anthocyanins' and their metabolites' effects on CYP450 isoforms other than CYP3A4 is invited to add to our understanding of polyphenolics' multiple functionalities and to promote the safe use of these compounds in food supplementation.

3.3.2 Effects on cytochrome P450 2D6

Amid growing safety concerns, the present study also addressed *in vitro* interactions with CYP2D6, an isoform highly relevant to the metabolism of many psychoactive drugs [459,604-607]. In continuation of earlier studies with grapefruit, green tea and grape seed extract [438,460] and the polyphenolic apple constituent quercetin [608,609], modulatory effects of 16 anthocyanins and anthocyanidins on cytochrome P450 2D6 activity were investigated, as was the effect of procyanidin B2.

Test compounds inhibited CYP2D6 activity in a concentration-dependent manner and determined IC_{50} values are summarized in table 3-8. Anthocyanidins' IC_{50} values ranged from 55 to 150 µM (mean 79 +/- 35 µM SD), and the four most potent inhibitors pelargonidin, peonidin, delphinidin and cyanidin emerged from this group. For anthocyanidin-monoglycosides IC_{50} values of 70 to 266 µM (mean 162 +/- 82 µM SD) were obtained. Finally, the least potent inhibitors were identified as the diglucosidic anthocyanins pelargonidin-3,5-diglucoside, cyanidin-3,5-diglucoside and procyanidin

B2, all of which featured IC_{50} values > 800 µM. Quinidine, a widely used inhibitor that had been selected as a reference, displayed an IC_{50} value of 6 nM.

Table 3-8 IC_{50} values calculated for inhibition of CYP2D6 activity by anthocyanidins, anthocyanins, procyanidin B2 and quinidine. Table adapted from [610], copyright 2009 Prous Science, S.A.U. or its licensors, all rights reserved.

Test compound	IC_{50} in µM (95% confidence interval)
Pelargonidin	55 (51-59)
Peonidin	59 (51-67)
Delphinidin	66 (65-68)
Cyanidin	69 (64-74)
Malvidin-3-glc	70 (68-72)
Malvidin-3-gal	73 (67-80)
Malvidin	77 (69-85)
Delphinidin-3-glc	109 (87-137)
Malvidin-3,5-diglc	145 (133-157)
Petunidin	150 (138-162)
Cyanidin-3-glc	151 (114-199)
Peonidin-3-glc	217 (199-237)
Cyanidin-3-gal	245 (217-278)
Cyanidin-3-rut	266 (169-420)
Pelargonidin-3,5-diglc	> 800 (-)
Cyanidin-3,5-diglc	> 800 (-)
Procyanidin B2	> 800 (-)
Quinidine	6 nM (5-7 nM)

Only few data are available on CYP2D6 inhibitory effects of defined polyphenolic compounds. Of these, quercetin exhibited an IC_{50} value of 24 µM [609], while flavonols and flavonol glycosides from wild ginger exhibited IC_{50} values ranging from 5 to 51 µM [611]. In contrast, furanocoumarins from grapefruit juice achieved 50% inhibition in the nanomolar range. Specifically, bergamottin, 6',7'-dihydroxybergamottin and the dimerics GF-I-1 (4-[[6-hydroxy-7-[[1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-*g*][1]benzopyran-7-one) and GF-I-4 (4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7H-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-*g*][1]benzopyran-7-one) reached IC_{50} values of 190 nM, 900 nM, 200 nM and 300 nM, respectively [438]. For comparison, psychoactive drugs yielded IC_{50}

values of 1.2 (paroxetine), 1.5 (perphenazine), 2.7 (thioridazine) and 6.5 nM (haloperidol) for CYP2D6 inhibition [612-614].

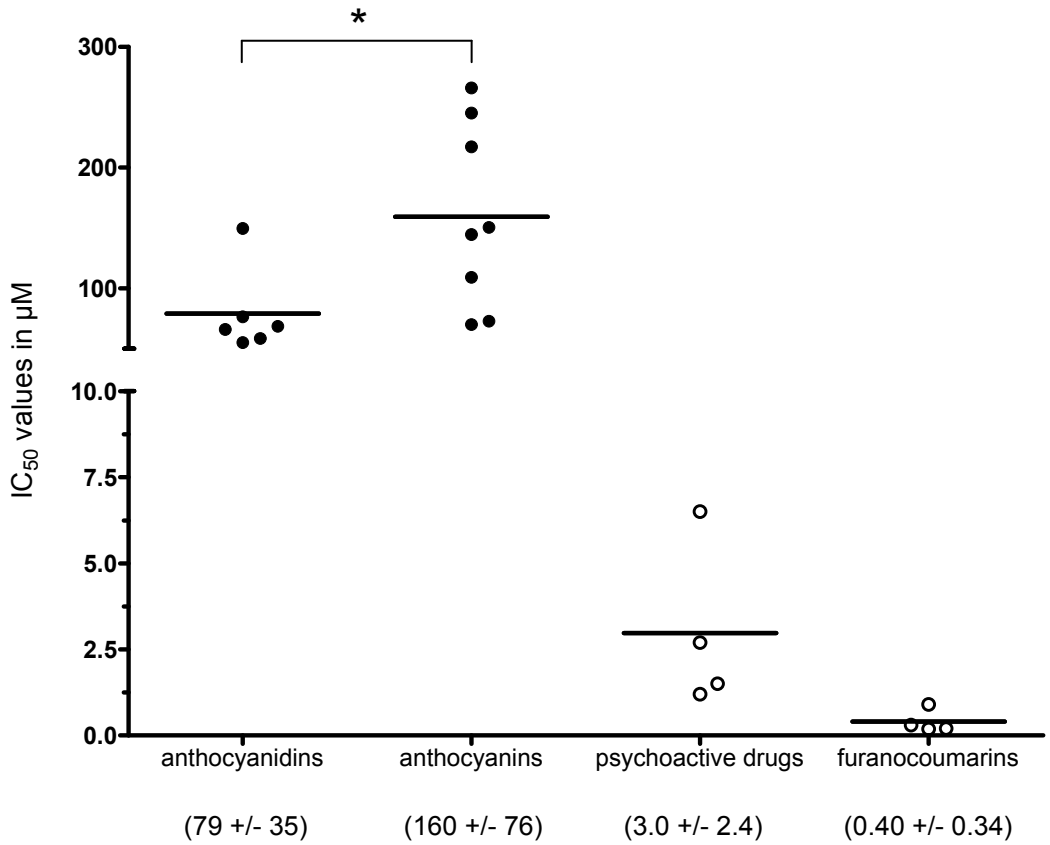

Figure 3-12 IC_{50} values of anthocyanidins, anthocyanins, psychoactive drugs (haloperidol, thioridazine, perphenazine, paroxetine) [612-614], and furanocoumarins [438]. Only those compounds are shown, for which IC_{50} values were available, i.e., excluding cyanidin-3,5-diglucoside and pelargonidin-3,5-diglucoside. Mean IC_{50} values +/- SD (µM) are given in brackets. Asterisk indicates difference between substance groups at $p < 0.05$. Figure adapted from [610], copyright 2009 Prous Science, S.A.U. or its licensors, all rights reserved.

Inhibitory effects of anthocyanins on CYP2D6 were weaker by several orders of magnitude, ranging from 70 µM to > 800 µM (figure 3-12). According to *in silico* prediction models, structural features required for effective CYP2D6 inhibition comprise a tertiary amine fragment plus a positive charge on nitrogen and flat hydrophobic region [615]. These ligand descriptors are not met by the compounds investigated here, and relative to the cinchona alkaloid quinidine, the inhibitory potential of anthocyanins and their aglycones was at least 10,000-fold lower. This suggests that anthocyanins pose a limited risk of food–drug interactions mediated by CYP2D6 as compared to the above-mentioned grapefruit, green tea and grape seed constituents, or other phytochemicals.

Following investigation of the modulatory effects of substances' sugar moieties on inhibitory potential, excluding cyanidin-3,5-diglc and pelargonidin-3,5-diglc for which IC_{50} values can not be calculated (> 800 µM), anthocyanidins were significantly better

CYP2D6 inhibitors than anthocyanins ($p = 0.0344$, $F = 5.695$, $t = 2.386$, $df = 12$). In a second approach, in addition to the presence of a sugar moiety, the numbers of B-ring substituents (2 or 3) and an interaction term (sugar moiety*substituents) are included in the model, excluding pelargonidin which is the only substance with one substituent on its B-ring. Again, a modulatory role of the sugar moieties is observed ($p = 0.0093$, $F = 10.86$). Fitting is similar when the sugar moiety*substituents interaction term is included in the model ($p = 0.0105$, $F = 10.39$), although the B-ring substitution pattern itself did not have a modulatory effect. Specifically, CYP2D6 inhibition decreased with more substituents on the B-ring of anthocyanidins, whereas it increased with more substituents on the B-ring of anthocyanins. Following Bonferroni correction for multiple testing, differences remain significant for anthocyanins with 2 B-ring substituents versus anthocyanidins with 2 B-ring substituents ($p = 0.0112$), anthocyanins with 2 substituents versus anthocyanins with 3 substituents ($p = 0.0158$) and anthocyanins with 2 substituents versus anthocyanidins with 3 substituents ($p = 0.0231$).

For an extrapolation of *in vitro* data to *in vivo* effects, additional parameters must be taken into account. Thus, CYP2D6-inhibitory effects have been observed for grapefruit furanocoumarins *in vitro* but not *in vivo* [438,616]. Similarly, *in vivo* effects of extracts rich in flavonol glycosides, biflavones and flavonolignans from *Ginkgo biloba* and milk thistle contrast with *in vitro* evidence of CYP2D6 inhibition [617-624]. It has also been proposed that CYP2D6 may be less susceptible to inhibition by food constituents than other isoforms, since substances must cross the intestinal barrier before they can act on hepatic CYP2D6, whereas high-level expression of other cytochromes in the intestine allows interactions during digestion [603].

In animal studies, anthocyanin concentrations of 0-640 nmol/kg have been obtained from liver tissue after oral administration [23,67,79,80,138,625]. As anthocyanin concentrations in the rat liver are suggested to be roughly at equilibrium with systemic plasma concentration for at least 30 min post ingestion [138], storage of anthocyanins in the liver is unlikely. If equilibrium of liver and systemic plasma concentrations is also assumed for the human body, anthocyanin concentrations in the liver may not reach the micromolar range at which CYP2D6 inhibition of 50% was observed *in vitro.* Therefore, depending on the *in vivo* portal availability of anthocyanins in humans, which has yet to be determined, the impact on metabolic processes may be even smaller than anticipated.

The clinical relevance of CYP450 inhibition is subject to individual differences in enzyme activity, i.e., the prevalence of functional genetic variants. The present data obtained with recombinant human CYP2D6 count against CYP2D6-modulatory effects of anthocyanins and anthocyanidins for subjects carrying the CYP2D6*1 allele [626],

i.e., for the majority of the Caucasian population. Whether similar effects can be expected in carriers of other CYP2D6 alleles remains to be determined.

As for the physicochemical underpinnings of anthocyanin–cytochrome interactions, molecular modelling investigations are encouraged to supplement the present exploratory approach to structure–function relationships.

Finally, characterization of anthocyanin effects on additional CYP450 isoforms is warranted to foster our understanding of the biological activity of berry polyphenolics. Thus, inhibitors of procarcinogen-activating isoforms (e.g. CYP1A or CYP1B) may limit the generation of harmful metabolites from estradiol, aromatic amines, heterocyclic amines and polycyclic aromatic hydrocarbons.

4 Material and methods

4.1 Chemicals

Unless noted otherwise, chemicals were obtained in p.a. quality from Sigma-Aldrich (Steinheim), Fluka (Deisenhofen), Carl Roth (Karlsruhe), Merck (Darmstadt), Fisher Scientific (Schwerte), ICN Biomedicals (Eschwege), USB (Staufen), Cayman Europe (Tallinn, Estonia), Extrasynthese (Genay, France).
Chemicals for cell culture experiments such as media, serums and antibiotics were purchased from Gibco (Eggenstein), Invitrogen (Karlsruhe), PAA (Cölbe) and PAN (Aidenbach), and Protease Inhibitor Cocktail was obtained from Sigma-Aldrich (Steinheim). Hoechst 33342, Ko143, calcein-AM, verapamil and sulfasalazine were provided by Solvo Biotechnology (Budaörs, Hungary) and radiochemicals were purchased from Perkin Elmer (Waltham, MA).
Ultrapure water was obtained upon filtration with Millipore cartridges (MilliQ biocel, Millipore, Schwalbach).

4.2 Consumables

Consumables were provided by Roth (Karlsruhe), Fisher-Scientific (Schwarte), Nunc (Wiesbaden), Nalgene (Hamburg), Sarstedt (Nümbrecht), Eppendorf (Hamburg), Falcon (Heidelberg), Becton Dickinson (Heidelberg), Biozym (Oldendorf) and VWR (Nürnberg).

4.3 Membrane preparations and enzymes

Membrane preparations from human BCRP transporter- and human MDR1 transporter-expressing *Spodoptera frugiperda* Sf9 ovarian cells, membrane vesicles in the inside-out orientation prepared from baculovirus-infected Sf9 cells overexpressing human BCRP transporters, Beta-gal and defBCRP membranes were supplied by SOLVO Biotechnology (Budaörs, Hungary).
Membrane preparations containing recombinant human CYP3A4 or CYP2D6 obtained from baculovirus expression systems were purchased from Promega (Mannheim). Recombinant human PLA_2-V was obtained from Cayman Europe (Tallinn, Estonia) and human MAO A and MAO B enzymes, expressed in baculovirus-infected insect cells (BTI-TN-5B1-4), were obtained from Sigma–Aldrich (Steinheim).

4.4 Cell lines

BCRP transporter-overexpressing MCF7-MX cells and MDR1-overexpressing K562-MDR cells were provided by Solvo Biotechnology (Budaörs, Hungary). HL-60 human acute myeloid leukaemia cells, SH-SY5Y human neuroblastoma cells and IMR-32 human neuroblastoma cells were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig).

4.5 Assay kits

CYP3A4 activity:	P450-Glo™ CYP3A4 Screening System (Luciferin-PPXE) DMSO Tolerant Assay (Promega, Mannheim)
CYP2D6 activity:	P450-Glo™ CYP2D6 Screening System (Promega, Mannheim)
HIF-1α ELISA:	Surveyor™ IC (R & D Systems, Wiesbaden)
HIF-2α ELISA:	HIF-2α ELISA Kit (Cusabio, Newark, DE)
MAO A and B activities:	MAO-Glo™ Assay (Promega, Mannheim)
Mycoplasma test:	Venor®GeM (Minerva Biolabs, Berlin)
Proteasome activity:	Proteasome-Glo™ Cell-Based Assay (Promega, Mannheim)
Protein quantification:	Pierce® Micoplate BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific, Bonn)

4.6 Instruments

4.6.1 Miscellaneous

Autoclave:	Varioklav steam sterilizer (H+P Labortechnik, Oberschleißheim)
Centrifuges:	Heraeus Megafuge 1.0, Megafuge 2.0R, Biofuge pico, Biofuge fresco (Heraeus, Hanau)
Compartment dryer:	WTB Binder (Binder, Tuttlingen)
Electrophoresis chamber:	PeqLab 40-1214 (PeqLab, Erlangen)
Fluorimeter:	BMG Labtech FluoStar Optima fluorescence photometer (BMG Labtech, Offenburg).
Gel documentation system:	MWG-Biotech (MWG Biotech, Ebersberg)
Incubator:	Hera cell (Heraeus, Hanau)

Luminometer:	Anthos lucy 1 microplate luminometer (Anthos, Wals/ Salzburg, Austria)
Lyophilizer:	Christ alpha 1-2 (Christ, Osterode)
Lyophilizer pump:	Rotary vane vacuum pump RZ 2 (Vacuubrand, Wertheim)
Microscope:	Olympus IX70 (Olympus, Hamburg)
Orbital Shaker:	IKA-VIBRAX-VXR Type VX7 (Janker & Kunkel, Staufen)
PCR cycler:	Trio Thermoblock Biometra (Biometra, Göttingen)
pH meter:	WTW pH 540 GLP (WTW, Weilheim)
Photometer:	Molecular Devices Emax precision microplate reader (MDS, Ismaning) Tecan Spectra Mini Photometer (Tecan, Crailsheim)
Scales:	Sartorius BL 1500, Sartorius BP 221S (Sartorius, Göttingen)
Scintillation counter:	Perkin Elmer MicroBeta Trilux liquid scintillation counter (Perkin Elmer, Waltham, MA)
Sterile workbench:	Hera safe Type HS9 (Heraeus, Hanau)

4.6.2 High performance liquid chromatography with diode array detection (HPLC-DAD)

HPLC system 1 (quercetin)

Instrument:	Photodiode array Hewlett Packard 1100 Series (Hewlett Packard, Waldbronn)
Pump:	Hewlett Packard high pressure pump for binary high pressure gradients, 1100 Series (Hewlett Packard, Waldbronn)
Autosampler:	Wisp 712b (Waters, Eschborn)
Column:	Synergy-Hydro-RP: 4.6 x 250 mm i.d., particle size 4 µm (Phenomenex, Aschaffenburg)
Eluent:	A 0.1% formic acid in H_2O B acetonitrile
Flow:	0.8 ml/min
Gradient:	linear, 1 - 45% B in 45 min
Injection:	25 µl
Detector:	Hewlett Packard Photodiode array (DAD), 1100 Series, 200-600 nm (Hewlett Packard, Waldbronn)
Wavelength:	360 nm

Software: Hewlett Packard ChemStation© Software (Hewlett Packard, Waldbronn)

HPLC system 2 (delphinidin, delphinidin-glc)

Instrument: Alexys 100 LC-EC System (Antec Leyden, Zouterwoude, Netherlands)
Pump: Alexys LC100 (Antec Leyden, Zouterwoude, Netherlands)
Autosampler: Alexys AS100 (Antec Leyden, Zouterwoude, Netherlands)
Column: Hypersil ODS: 4.0 x 125 mm i.d., particle size 5 µM (Thermo Fisher Scientific, Dreieich)
Eluent: A 10% formic acid in H_2O (v/v): acetonitrile (1:1, v/v)
B 10% formic acid in H_2O (v/v)
Flow: 0.5 ml/min
Gradient:

Time (min)	Eluent B (%)
0	94
40	78
60	56
63	6
68	6
71	94
80	94

Injection: 100 µl
Detector: SPA-10A UV-Vis Detector (Shimadzu, Duisburg)
Wavelength: 520 nm
Software: Alexys data system (Antec Leyden, Zouterwoude, Netherlands)

4.6.3 High performance liquid chromatography mass spectrometry and tandem mass spectrometry (HPLC-MS, HPLC-MS/MS)

HPLC system 3 (quercetin, gallic acid, phloroglucinol)

HPLC: Photodiode array Hewlett Packard 1100 Series (Hewlett Packard, Waldbronn)

Pump:	Hewlett Packard high pressure pump for binary high pressure gradients, 1100 Series (Hewlett Packard, Waldbronn)
Autosampler:	Triathlon (Spark, Emmen, Netherlands)
Column:	Symmetry C18: 2.1 x 150 mm, particle size 5 µm (Waters, Eschborn)
Eluent:	A 0.1% formic acid in H_2O
	B acetonitrile
Flow:	0.2 ml/min
Gradient:	linear, 1 - 40% B in 40 min
Injection:	25 µl
Detector:	Hewlett Packard Photodiode array (DAD), 1100 Series, 200-600 nm (Hewlett Packard, Waldbronn)
Software:	Hewlett Packard ChemStation© Software (Hewlett Packard, Waldbronn)
Instrument:	Finnigan TSQ 7000 Triple-stage quadrupol tandem mass spectrometer (Finnigan MAT, Bremen) with Finnigan electrospray ionisation interface (ESI)
ESI capillary:	deactivated fused-silica capillary: 50 µm i.d (J&W Scientific, Folsom, CA)
Ion source:	atmospheric pressure, room temperature
Inlet capillary:	250 °C
Modus:	negative
Capillary voltage:	3.2 kV
Sheath gas:	N_2 5.0 (70 psi)
Auxiliary gas:	N_2 5.0 (10 l/min)
Scan range:	*m/z* 150 - 1000 (Full scan mode)
Collision gas:	Argon 5.0, pressure 0.27 Pa (2.0 mTorr)
Collision energy:	20 - 30 eV
Multiplier voltage:	1600 V (ESI-MS), 1950 V (ESI-MS/MS)
Software:	Xcalibur™ Software Version 1.2 (Thermo Electron, Dreieich)

HPLC system 4 (delphinidin, delphinidin-glc)

HPLC:	Photodiode array Hewlett Packard 1100 Serie (Hewlett Packard, Waldbronn)
Pump:	Hewlett Packard high pressure pump for binary high pressure gradients, 1100 Series (Hewlett Packard, Waldbronn)

Autosampler:	Triathlon (Spark, Emmen, Netherlands)
Column:	Symmetry C18: 2.1 x 150 mm, particle size 5 µm (Waters, Eschborn)
Eluent:	A 1% formic acid in H_2O
	B acetonitrile
Flow:	0.2 ml/min
Gradient:	linear, 1 - 40% B in 40 min
Injection:	25 µl
Detector:	Hewlett Packard Photodiode array (DAD) 1100 Series, 200-600 nm (Hewlett Packard, Waldbronn)
Wavelength:	520 nm
Software:	Hewlett Packard ChemStation© Software (Hewlett Packard, Waldbronn)
Instrument:	Finnigan TSQ 7000 Triple-stage quadrupol tandem mass spectrometer (Finnigan MAT, Bremen) with Finnigan electrospray ionisation interface (ESI)
ESI capillary:	deactivated fused-silica capillary: 50 µm i.d (J&W Scientific, Folsom, CA)
Ion source:	atmospheric pressure, room temperature
Inlet capillary:	250 °C
Modus:	positive
Capillary voltage:	3.5 kV
Sheath gas:	N_2 5.0 (70 psi)
Auxiliary gas:	N_2 5.0 (10 l/min)
Scan range:	*m/z* 150 - 1000 (Full scan mode)
Collision gas:	Argon 5.0, pressure 0.27 Pa (2.0 mTorr)
Collision enery:	20 eV
Multiplier voltage:	1600 V (ESI-MS), 1950 V (ESI-MS/MS)
Software:	Xcalibur™ Software Version 1.2 (Thermo Electron, Dreieich)

4.7 Programs

ISIS/Draw V2.1.4 (MDL Information Systems, CA, USA) served to illustrate chemical structures. Prism V 4.00 (GraphPad Software, La Jolla, CA, USA) was applied for generation of dose-response curves and for calculations of IC_{50} and EC_{50} values. Analyses of variance were performed with Prism V 4.00 and Statistica V 8.0 (StatSoft,

Tulsa, OK, USA). Microsoft Office Excel 2003 (Microsoft Corporation, WA, USA) was used for enzyme kinetics analyses.

4.8 Methods

4.8.1 General proceedings with cell lines

4.8.1.1 Thawing

Ampules with respective cells were collected from liquid nitrogen storage and placed in a 37 °C water bath. They were allowed to thaw until a small amount of ice remained. Cells were slowly pipetted into 5 ml of icecold culture medium and centrifuged at 1,200 rpm for 5 min. After supernatant was discarded, the cell pellet was resuspended in warm medium and transferred to a cell culture flask. The following day, culture medium was changed.

4.8.1.2 Culture

HL-60 and SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin plus 100 µg/ml streptomycin, and IMR-32 cells were cultured in RPMI-1640 supplemented with 10% FCS, 100 units/ml penicillin plus 100 µg/ml streptomycin. Cells were maintained in an atmosphere of 5% CO_2 at a temperature of 37 °C. Depending on the cell line, passages were carried out every three to five days.

Passage of suspension cells

An aliquot of HL-60 cells in suspension were diluted with fresh medium. After removal of spent medium, weakly adherent IMR-32 cells were carefully washed with phosphate-buffered saline (PBS), rinsed off with fresh medium and the obtained suspension was further diluted with culture medium after a cell count was performed.

Passage of adherent cells

For SH-SY5Y cells, spent medium was aspirated and cells were washed with PBS. The cell monolayer was covered with trpsin/EDTA (1 ml/25 cm² flask) and incubated for 1-2 min at 37 °C. Remaining attached cells were released by gently tapping the flask, followed by resuspension in 7 ml fresh medium. Upon centrifugation at 1,200 rpm for 5 min, supernatant was discarded and an aliquot of cells was resuspended in fresh culture medium after determination of cell number.

4.8.1.3 Freezing

For long-term storage, cells were cryoconserved in liquid nitrogen at -196 °C. Where applicable, cells were detached and suspended, separated from the spent medium by centrifugation at 1,200 rpm for 3 min, the supernatant was discarded and the cell pellet was resuspended in 500 µl of icecold fresh medium. Then 500 µl of freezing medium (FCS: dimethyl sulfoxide (DMSO) = 9:1) was added and resuspended cells were filled into cryovials. Cells were gently frozen applying a two-step procedure. After vials were stored in a cryobox filled with isopropanol at -70 °C for 24 h, guaranteeing a uniformly decreasing temperature, they were transferred into liquid nitrogen for long-term storage.

4.8.1.4 Determination of cell number and vitality

Cell numbers were determined with a Neubauer counting chamber. Therefore 50 µl cell suspension was mixed with 50 µl trypan blue solution (0.5%) and 100 µl PBS, and an aliquot of the mixture was fed into the counting chamber. As the trypan blue dye can permeate damaged cell membranes, death cells appear blue under the microscope.
Since the area of one large square amounts to 1 mm², considering the 0.1 mm depth of the chamber the volume under one large square amounts to 0.1 µl. All four large squares were counted and the cell number calculated as follows:

$$\text{Cells/ml} = \frac{\text{n} \cdot D}{0.0004\ \text{ml}}$$

where n is the number of cells counted and D the dilution factor.

4.8.1.5 Mycoplasma test

Mycoplasma test Venor®GeM was performed according to the manufacturer's protocol. Briefly, 100 µl supernatant of confluent cell cultures was transferred to a tightly sealed tube and incubated at 95 °C for 5 min. Following centrifugation at 13,000 rpm for 5 sec to pellet cellular debris, tubes were kept on ice prior to use for polymerase chain reaction (PCR). For PCR reaction, total volume per reaction was 25 µl, composed as follows:

Reagent	Volume (µl)
PCR grade water	15.3
10 x Reaction buffer	2.5
Primer/Nucleotide mix	2.5

Internal control	2.5
Sample DNA / Positive control DNA / Negative control (water)	2
Taq polymerase	0.2

When setting up reactions, positive and negative controls were always included and the following PCR program was applied:

1 cycle		94 °C	2 min
35 cycles	Denaturation	94 °C	30 sec
	Annealing	55 °C	30 sec
	Elongation	72 °C	30 sec

Results were analyzed after gel electrophoresis, where a 191 bp band was seen as internal control band and a 267 bp band was visible in the positive control lane.

4.8.2 Interactions with ABC efflux transporters

4.8.2.1 Assays

4.8.2.1.1 Dye extrusion assay

For assessing effects on BCRP, Hoechst fluorescent dye 33342 was added to BCRP transporter-overexpressing MCF7-MX cells. Modulators of BCRP transporter activity reduce the rate of Hoechst 33342 extrusion and cause its accumulation inside the cells. Following DNA intercalation, a fluorescent signal may be detected that is proportional to BCRP transporter inhibition. Briefly, cells ($1x10^5$ per well of standard 96-well tissue culture plates) were incubated with Hoechst 33342 (50 µM) plus the test compound or, alternatively, with DMSO for non-inhibited controls, or Ko143 (1 µM) as a reference inhibitor, at 37 °C for 15 min in Hank's Balanced Salt Solution (HBSS). Fluorescence of accumulated Hoechst 33342 inside cells was measured in real time at excitation and emission wavelengths of 355 and 460 nm, respectively.

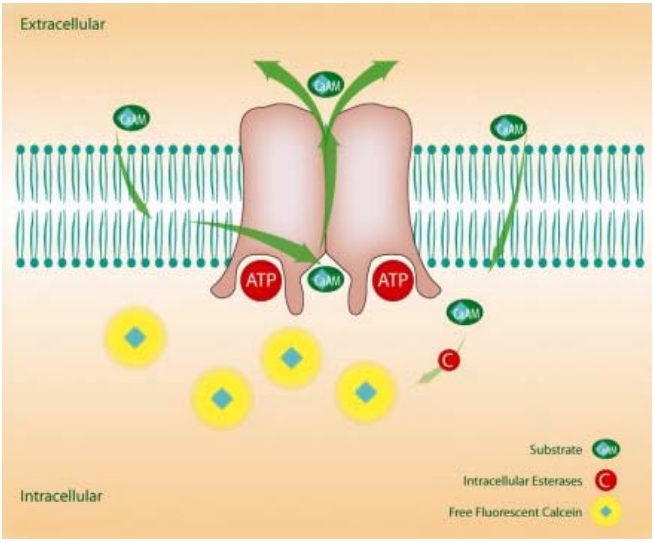

Figure 4-1 Principle of the dye extrusion assay for MDR1. Image courtesy of SOLVO Biotechnology (www.solvo.com, 2011).

For quantification of effects on MDR1, calcein-AM was added to MDR1-overexpressing K562-MDR cells. Modulators of MDR1 activity cause accumulation of calcein-AM inside the cell, whereupon it is cleaved by non-specific esterases to form the fluorescent low permeability dye calcein (figure 4-1). The increase of fluorescent signal inside the cell is proportional to MDR1 inhibition. Briefly, K562-MDR cells ($8x10^4$ cells per well) and calcein-AM (0.25 µM) were incubated with the test compound or, alternatively, with DMSO for non-inhibited controls, or verapamil (60 µM) as a reference inhibitor, at 37 °C for 8 min in HBSS. Fluorescence of accumulated calcein inside cells was measured at excitation and emission wavelengths of 485 and 538 nm, respectively. After the incubation period, propidium iodide was added to all wells (0.01 mg/ml) and fluorescence was measured at 530 and 630 nm excitation and emission wavelengths, respectively, to assess potential cytotoxic effects of the substances tested.

4.8.2.1.2 ATPase assay

ATPase activity of wild-type human BCRP and MDR1 was measured, according to a protocol modified from [465], by colorimetric detection of inorganic phosphate, the byproduct of ABC transporter function (figure 4-2). Membrane preparations from human BCRP transporter- and human MDR1 transporter-expressing *Spodoptera frugiperda* Sf9 ovarian cells were applied.

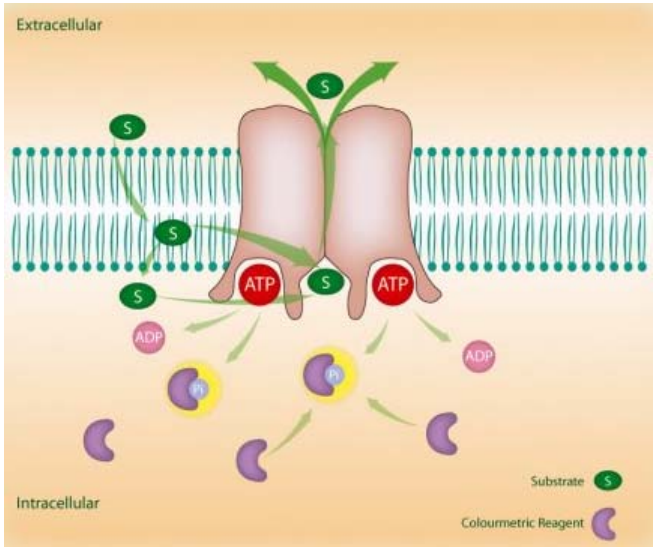

Figure 4-2 Principle of the ATPase assay. Image courtesy of SOLVO Biotechnology (www.solvo.com, 2011).

For assessing BCRP and MDR1 transporter-related ATPase activity, incubations were carried out in the presence and absence of sodium *o*-vanadate (Na_3VO_4) (1.2 mM), a known ABC transporter inhibitor, to distinguish between background ATPase activity and transporter-related ATPase activity. The ATPase assay was performed according to the manufacturer's protocol. Briefly, membranes (20 µg/well) were preincubated at 37 °C for 10 min with the test substance or the solvent DMSO in the absence and presence of vanadate, and reactions were started by adding MgATP. Sulfasalazine at 10 µM and verapamil at 40 µM served for activation of BCRP and MDR1 membranes, respectively. Final concentrations of assay compounds amounted to 10 mM $MgCl_2$, 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)-Tris (pH 7.0), 50 mM KCl, 5 mM dithiothreitol, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 4 mM sodium azide, 1 mM ouabain, and 5 mM ATP. Reactions were stopped after 10 min at 37 °C by the addition of the color-developing agent followed by a blocking reagent. Another 30 min later, absorbance at 620 nm was detected using a fluorescence photometer. Phosphate standards (0.4 and 0.8 pmol/well) for optical density (OD) calibration were included on each 96-well plate. For calculation of relative ATPase activity, baseline ATPase activity (ctrl 1), Na_3VO_4-insensitive ATPase activity (ctrl 2), ATPase activity of fully activated membranes (ctrl 3), and Na_3VO_4-insensitive ATPase activity of fully activated membranes (ctrl 4) were also determined. Beta-gal and defBCRP membranes served as negative controls for MDR1 and BCRP, respectively.

4.8.2.1.3 Vesicular transport assay

Vesicular transport assays were performed as previously described [627], using membrane vesicles in the inside-out orientation prepared from baculovirus-infected Sf9 cells overexpressing human BCRP transporters.

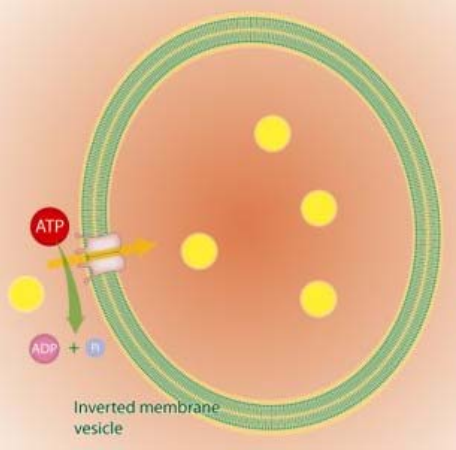

Figure 4-3 Principle of the vesicular transport assay. Image courtesy of SOLVO Biotechnology (www.solvo.com, 2011).

^{3}H-estrone-3-sulfate was used as a radiolabeled, low-permeability reporter substrate which is transported into the vehicles by the transporters (figure 4-3). Incubations were carried out in the absence and presence of 4 mM ATP to distinguish between transporter-related uptake and passive diffusion into the vesicles. The reference inhibitor Ko143 (300 nM) served as a positive control and defBCRP as negative control. Briefly, membrane vesicle preparations were preincubated with reporter substrate and the test substance or, alternatively, the solvent DMSO at 37 °C for 10 min in assay buffer, containing 10 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.0) and 250 mM sucrose. Addition of MgATP, or assay buffer for background controls, started the reaction. After one min, reactions were stopped by the addition of icecold assay buffer. Vesicles were separated by immediate filtration on a 96-well filter plate (0.65 µm, Millipore, Billerica, MA), filter plates were washed, dried and reporter substrate inside the filtered vesicles was quantified by liquid scintillation.

Stock solutions of the flavonoids (15 mM) and serial dilutions were prepared in DMSO. Final assay concentrations were 0.07, 0.21, 0.62, 1.85, 5.55, 16.67, 50, 150 µM for the dye extrusion assays and 0.41, 1.2, 3.7, 11, 33, 100, 300 µM for ATPase and vesicular transport assays. For each concentration step tested, assays were performed in triplicate.

4.8.2.2 Data analysis

GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA) was used for curve fitting, determination of reaction parameters and statistical analysis.

4.8.2.2.1 Dye extrusion assay

The rate of dye accumulation was defined as the slope fitted onto the data points of the fluorescence versus time plot. For calculation of relative transporter inhibition (%), values from DMSO and control inhibitors (Ko143 and verapamil) were defined as 0% and 100% inhibition, respectively, by the following equation:

$$\frac{\text{flavonoid} - \text{DMSO}}{\text{control inhibitor} - \text{DMSO}} \cdot 100$$

IC_{50} was defined as the test substance concentration required for inhibiting the transport of the reporter substrate by 50%. Efficacy describes the maximal inhibition achieved by a test compound in percent of the maximal inhibition observed in the presence of the reference inhibitors Ko143 and verapamil.

4.8.2.2.2 ATPase assay

For calculation of relative activation and inhibition, the vanadate-sensitive baseline ATPase activity and the maximal vanadate-sensitive ATPase activity after stimulation with 10 µM sulfasalazine, for BCRP, or 40 µM verapamil, for MDR1, were defined as 0% and 100% transporter ATPase activity, respectively. Relative activation (%) was calculated as follows:

$$\frac{[(\text{flavonoid} - (\text{flavonoid} + \text{vanadate})) - (\text{ctrl1} - \text{ctrl2})]}{(\text{ctrl3} - \text{ctrl4}) - (\text{ctrl1} - \text{ctrl2})} \cdot 100$$

EC_{50} was defined as the test substance concentration needed to reach 50% of its own maximal activation and efficacy was defined as the compounds' maximal activation, as compared to the activation by the respective reference. Relative inhibition values (%) were obtained as follows:

$$\frac{[((\text{flavonoid} + \text{reference activator}) - (\text{flavonoid} + \text{reference activator} + \text{vanadate})) - (\text{ctrl1} - \text{ctrl2})]}{(\text{ctrl3} - \text{ctrl4}) - (\text{ctrl1} - \text{ctrl2})} \cdot 100$$

IC_{50} was defined as the test substance concentration at which half-maximal inhibition occurred and efficacy was defined as the maximal inhibitory effect achieved by the test substance relative to the baseline activity.

4.8.2.2.3 Vesicular transport assay

Substrate transport relative to the non-inhibited control (%) was calculated according to the following equation:

$$\frac{(\text{flavonoid} + \text{ATP}) - \text{flavonoid}}{(\text{DMSO} + \text{ATP}) - \text{DMSO}} \cdot 100$$

IC_{50} was defined as the test substance concentration required for inhibiting transport of the reporter substrate by 50%. Efficacy specifies the maximal inhibition achieved by the test compound in percent of the maximal activity.

4.8.2.2.4 Statistics

To address a putative structural effect of tested anthocyanins' sugar component on BCRP ATPase activation and vesicular transport, substances were grouped by the presence or absence of sugar moieties, i.e. anthocyanins versus anthocyanidins. EC_{50} and IC_{50} values, respectively, were compared with an unpaired t-test following confirmation of Gaussian distributions. To correct for unequal variances (F-test), Welch's correction was performed. Statistical significance was set at $p = 0.05$.

4.8.3 Interactions with monoamine oxidases A and B

4.8.3.1 Monoamine oxidases A and B activity assay

Effects of test substances on MAO activities were determined using the MAO-Glo™ chemiluminescent assay according to the manufacturer's protocol (table 4-1).

Table 4-1 MAO assay protocol.

Reagent	Volume (µl)	Incubation time (min) MAO A / B
Substrate solution	12.5	
MAO buffer	11.5	
Test substance / DMSO	1	
Enzyme solution in buffer / buffer	25	90 / 120
Luciferin detection reagent	50	30

Briefly, MAO substrate was incubated for 90 (MAO A) and 120 min (MAO B) at room temperature with the test compounds and the respective MAO enzyme (20 µg/ml). Assays were performed in 96-well microtiter plates in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer. Based on preliminary investigations, compounds' final concentrations in the assay were chosen to range from 4 to 400 µM for anthocyanins and anthocyanidins, from 100 to 1,000 or 2,000 µM (MAO A) and from 20 to 400 µM (MAO B) for procyanidins, from 400 to 25,000 µM for phenolic acids, from 100 pM to 2 µM for clorgyline, and from 0.1 to 10 µM for R-(-)-deprenyl. Since test compounds were dissolved and diluted with DMSO, an equivalent volume of DMSO was used as a negative control. MAO substrate, an aminopropylether analog of luciferin methyl ester, was used at concentrations of 40 µM and 4 µM, corresponding to K_m values determined by the manufacturer for MAO A and MAO B, respectively. To initiate a luminescent signal, the detection reagent was added, containing an esterase and a luciferase, and after another 30 min, chemiluminescence values, displayed as relative light units (RLUs), were recorded with a measuring time of 12 s for each well.
Since cyanidin-glycosides are by far the most prevalent anthocyanins in fruits, the kinetics underlying the effects of cyanidin and cyanidin-3-glucoside on the oxidative deamination of a MAO substrate by MAO A and MAO B were studied. Inhibition was measured at test compound concentrations of 20, 40 and 80 µM for cyanidin and 20, 40 and 80 µM (MAO A) plus 40, 80 and 120 µM (MAO B) for cyanidin-3-glucoside. For each test compound concentration, five concentrations of luminogenic substrate were used, i.e. 12.5-150 µM (MAO A) and 2-40 µM (MAO B), and an equivalent volume of buffer as a non-enzyme control.

4.8.3.2 Data analysis

For quantification of MAO inhibition, enzyme activity was calculated using the light signal generated by oxidation of D-luciferin which, in turn, is produced by MAO deamination of the substrate and subsequent spontaneous hydrolysis and β-elimination reactions (figure 4-4).

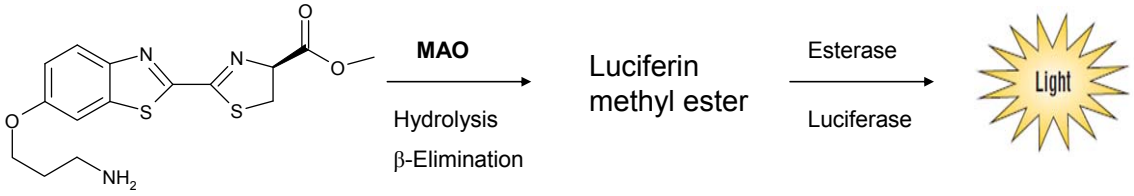

Figure 4-4 MAO catalyzed and subsequent reactions.

As the amount of light is directly proportional to the amount of luciferin released in the reaction with MAO enzyme, the following equation applies:

$$\%A = 100 \times \frac{A_I}{A_{DMSO}}$$

where %A is the percentage of the MAO activity remaining after the exposure to test substances, A_I is the activity in the presence of an inhibitor, and A_{DMSO} is the enzyme activity in the absence of inhibitors.

For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to seven concentration steps, using a non-linear regression model to determine the concentration inhibiting 50% of maximum MAO activity. Inhibition kinetics of cyanidin and the corresponding glucoside were analyzed by Lineweaver-Burk plots. K_i values were determined from secondary plots of the inhibitor concentration [I] versus the slope in Lineweaver-Burk plots. Mean values were formed from two separate experiments performed in duplicate.

To address a putative structural effect of test anthocyanins' sugar component, substances were grouped by the number of sugar moieties, i.e. (i) anthocyanins, (ii) anthocyanidin-3-glycosides, and (iii) anthocyanidin-3,5-diglucosides for analysis of variance (Prism V4.00, GraphPad Software, CA, USA). Statistical significance was set at $p = 0.05$.

4.8.4 Influence on HIF-1α and HIF-2α expression

4.8.4.1 Cell incubation with test substances

All HIF-α expression experiments were performed on SH-SY5Y and IMR-32 neuroblastoma cells, cultivated as described in section 4.8.1.2. In a first round of experiments, cells were incubated with various test substances. Since phorbol-12-myristate-13-acetate (PMA) increased HIF-1α expression in preliminary experiments, it was employed throughout all experiments. PMA and compounds under study were dissolved and diluted with DMSO, with the exception of desferrioxamine which was dissolved in H_2O. When cells reached half to three-quarter confluency, fresh medium was added to culture dishes, containing 0.01% PMA and the respective substance to be tested. Under these conditions, cells were incubated at 37 °C for 4 h. Final substance concentrations amounted to 100 µM for anthocyanins, anthocyanidins, phenolic acids, procyanidin B2, quercetin and DFO, while bilberry extract was applied at 50 mg/l. DMSO concentrations were adjusted to 0.1% for all experiments. All incubations were performed in duplicate in both cell lines.

Based on findings during incubation experiments with these test substances, experiments were continued with delphinidin. In a second and third level of experiments, time- and concentration-dependent effects were under investigation. Therefore, incubations were performed with varying incubation times of 3, 4 and 5 h and with delphinidin concentrations of 25, 50 and 100 µM.

4.8.4.2 MTT cell viability assay

Moreover, the present work aimed to determine potential harmful effects test substances may exert on cells upon incubation experiments. As the reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation, the MTT assay was applied to study cytotoxicity of test compounds on SH-SY5Y cells. The yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by metabolically active cells, in part by the action of mitochondrial dehydrogenase enzymes, to generate reducing equivalents. The resulting intracellular purple formazan dye is directly related to the number of live cells and can be solubilized and quantified spectrophotometrically (figure 4-5).

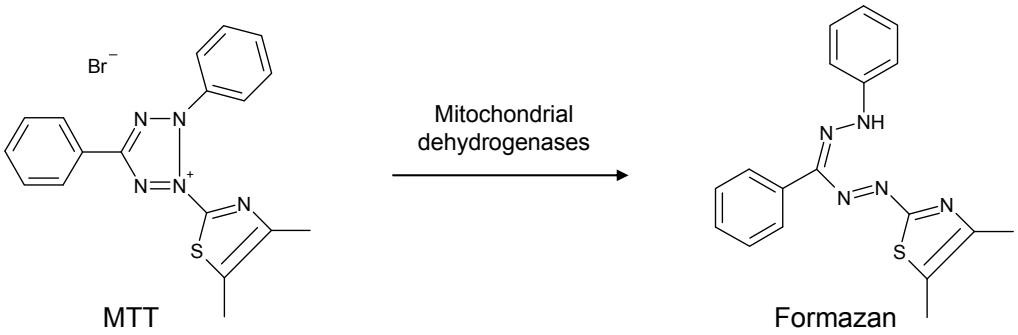

Figure 4-5 MTT assay: In metabolically active cells, MTT is converted to a formazan dye by mitochondrial dehydrogenase enzymes.

On the day prior to assay performance, SH-SY5Y cells were transferred to 96-well plates at a density of 3.6×10^4 cells/well in phenol red free RPMI medium supplemented with 10% FCS, 100 units/ml penicillin, plus 100 µg/ml streptomycin. After cells were allowed to attach for 24 h, test substances diluted with the same RPMI medium were added to yield final concentrations of 91 µM (bilberry extract 46 mg/l), with DMSO concentrations of 0.09%. DMSO in RPMI medium served as control, as did incubations for all test substances without cells aiming to subtract compounds distinct absorptive properties. After 4 h of incubation at 37 °C, an MTT solution prepared with PBS was added at a final concentration of 0.5 mg/ml, followed by another 3 h period of incubation at 37 °C. Medium was carefully removed to avoid cell detachment from

surface and intracellularly generated crystal needles of formazan were dissolved in DMSO-ethanol (4:1). Upon complete formazan solution, absorption was measured photometrically at 540 against 650 nm. For each compound tested, MTT assay was performed in duplicate and corrected values were compared to the control value.

Table 4-2 MTT assay protocol.

Reagent	Volume (µl)	Incubation time (h)
Cell suspension	100	24
Test substance solution	10	4
MTT solution	12.2	3
DMSO-ethanol solution	120	

To address effects of substances on cell viability compared to control values, analysis of variance was performed (Prism V 4.00, GraphPad Software, La Jolla, CA, USA, Statistica V 8.0, StatSoft, Tulsa, OK, USA). As all compounds' effects were compared to the same control group, Dunnett test was applied to correct p values for multiple comparisons.

4.8.4.3 Preparation of protein lysates

Following incubation with test substances, cells were detached, lysed and a protein extract was prepared. Therefore culture medium was removed and cells were carefully washed (at least three times) with warm PBS. Upon addition of icecold PBS, SH-SY5Y cells were scraped off and IMR-32 cells were rinsed off the dish and transferred to centrifugation tubes. After this suspension was centrifuged at 500 rpm for 5 min at 4 °C, pelleted cells were resuspended in 30 or 200 µl lysis buffer, depending on the experimental size. Lysis buffer was composed as follows and adjusted to pH 7.9 with HCl:

Substance	Concentration
NaCl	300 mM
Tris	10 mM
EDTA	1 mM
Nonidet P-40	0.1% (v/v)
Protease Inhibitor Cocktail	1%

This lysis buffer had the advantage over two concurring, reducing agent dithiothreitol containing lysis buffers since it achieved high protein recoveries and showed minimal interaction with assays for determination of protein and HIF-1α concentrations.
Cell suspensions were vortexed at maximal speed for 10 sec, tubes were placed in an icecold rack and put on an orbital shaker for 20 min, vortexed at maximal speed for 30 sec and centrifuged at 9,000 rpm for 10 min at 4 °C. Finally the protein containing supernatant was carefully separated from the cell debris pellet and immediately stored at -80 °C.

4.8.4.4 Determination of protein concentrations

Protein quantification in cell lysates was performed with the BCA protein assay according to the manufacturer's instructions (table 4-3). This assay is based on the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium and the subsequent sensitive and selective colorimetric detection of the cuprous cation chelate complex with bicinchoninic acid (BCA) (figure 4-6).

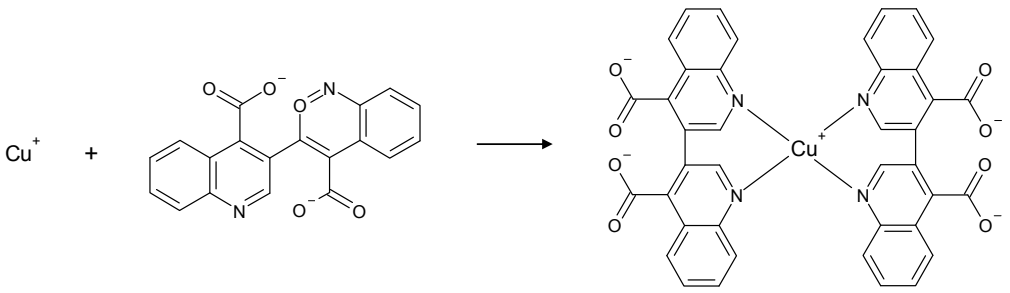

Figure 4-6 BCA protein assay: Chelation of BCA with the cuprous ion results in an intense purple color.

Briefly, samples or bovine serum albumine (BSA) protein standards were incubated with assay reagents, containing NaOH, Na_2CO_3, $NaHCO_3$, $CuSO_4$, bicinchoninic acid, and sodium tartrate, for 30 min at 37 °C and upon cooling to room temperature, absorption at 540 nm was measured photometrically. BSA standards were used at five concentrations ranging from 25 to 500 µg/ml. All samples were diluted with 0.9% NaCl containing 0.05% NaN_3, which also served as control sample.

Table 4-3 BCA assay protocol.

Reagent	Volume (µl)	Incubation time (min)
Sample / BSA standard solution	9	
Compatibility reagent solution	4	30
Working reagent	260	30

To exclude possible interactions of the lysis buffer with the assay, lysis buffer in standard samples and control samples was adjusted to concentrations in the test samples. Interference of other extract components with the assay demanded highest possible dilution of samples. For each sample, protein content was determined in duplicate. A standard curve was generated by plotting the corrected net absorptions for BSA standards versus its concentrations, and the resulting equation was applied to calculate protein concentrations of samples.

4.8.4.5 Determination of HIF-1α concentrations

HIF-1α concentrations of cellular protein extracts were determined with the Surveyor™ IC Immunoassay according to the manufacturer's protocol (table 4-4). This assay employs a two-site sandwich enzyme-linked immunosorbent assay (ELISA) and was performed at room temperature (figure 4-7).

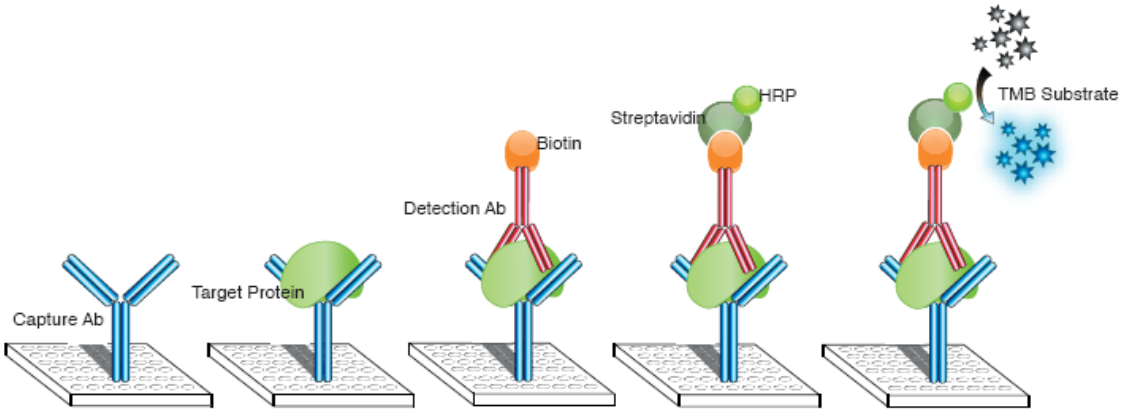

Figure 4-7 Principle of HIF-1α ELISA. Image courtesy of Epitomics, Inc. (www.epitomics.com, 2011).

An antibody specific for HIF-1α has been precoated onto a microplate, standards and samples were added and HIF-1α present was bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing HIF-1α was used to detect HIF-1α utilizing a standard streptavidin-horseradish peroxidase (HRP) format. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to the wells and color developed in proportion to the amount of HIF-1α present in the

sample. Color development was stopped and absorption was measured photometrically at 450 against 540 nm.

Table 4-4 HIF-1α ELISA protocol.

Reagent	Volume (µl)	Incubation time (min)
Test substance / standard solution	100	120
Remove solution, wash buffer	3 x 400	
HIF-1α detection antibody	100	120
Remove solution, wash buffer	3 x 400	
Streptavidin-HRP solution	100	120
Remove solution, wash buffer	3 x 400	
TMB substrate solution	100	20
Stop solution	50	

HIF-1α standards were used at 8 concentrations, ranging from 40 to 8,000 pg/ml. Samples were adjusted to uniform protein contents, ranging from 43 to 100 µg/ml for SH-SY5Y and from 100 to 150 µg/ml for IMR-32 incubations. For each sample, HIF-1α concentration was determined in duplicate. A nonlinear regression model was used to create standard curves and to calculate sample HIF-1α concentrations.

4.8.4.6 Determination of HIF-2α concentrations

HIF-2α concentrations of cellular protein extracts were determined with the Human HIF-2 ELISA Kit according to the manufacturer's protocol (table 4-5). The sandwich ELISA principle corresponds to the one described for HIF-1α, except that avidin-HRP was applied instead of streptavidin-HRP and the performance at 37 °C.

Table 4-5 HIF-2α ELISA protocol

Reagent	Volume (µl)	Incubation time (min)
Test substance / standard solution	100	120
Remove solution		
HIF-2 α detection antibody	100	60
Remove solution, wash buffer	3 x 400	
Avidin-HRP solution	100	60
Remove solution, wash buffer	3 x 400	
TMB substrate solution	90	30
Stop solution	50	

HIF-2α standards were used at 7 concentration steps ranging from 156 to 10,000 pg/ml. Samples were adjusted to the same protein content of 142 µg/ml for SH-SY5Y and of 300 µg/ml for IMR-32 incubations. Since the lysis buffer interfered with the assay, its concentrations in samples and standards were equalised and kept at a minimum by sample dilution. For each sample, protein content was determined in duplicate. A nonlinear regression model was used to create standard curves and to calculate sample HIF-2α concentrations.

4.8.4.7 HIF statistics

To address modulatory effects of test compounds on HIF-1α and HIF-2α expression, analysis of variance was performed (Prism V 4.00, GraphPad Software, La Jolla, CA, USA, Statistica V 8.0, StatSoft, Tulsa, OK, USA). Since all test substances' effects were compared to the same control, Dunnett test was applied to correct for multiple comparisons. When incubation time-dependent variations in modulatory effects on HIF-1α for delphinidin were monitored, t-tests were run for each incubation time comparing delphinidin effects to the respective controls'. To consider concentration-dependent effects of delphinidin on HIF-1α expression, ANOVA followed by Dunnett correction were used.

4.8.4.8 Cell uptake of substances under study

To address uptake of compounds with modulatory effects on HIF-1α expression by SH-SY5Y and IMR-32 cells, recoveries of quercetin, delphinidin and delphinidin-glc from cell lysates were investigated and compared to control incubations. Therefore cells were incubated with substances as described in chapter 4.8.4.1. Then cells were washed five times with warm PBS to prevent that extracellular test substances get into cell lysates and falsify results. After cells lysis according to section 4.8.4.3, an aliquot was used for quantification of protein content and the remaining lysate was adjusted to pH 1 with 1N HCl to favor stability of test substances. Upon freezing and lyophilisation of samples, dried extracts were thrice extracted with methanol containing 5% formic acid. Samples were centrifuged at 1,000 rpm for 10 min and supernatants were separated and dried under a stream of nitrogen. While quercetin containing samples were redissolved in 50% methanol and 3,4,5-trihydroxycinnamic acid was added as an internal standard, the remaining extracts were redissolved in 10% formic acid. These solutions were subsequently used for HPLC analyses.

To investigate recovery of native test substances, HPLC-VIS analyses were performed detecting quercetin absorbance at 360 nm (system 1) and delphinidin and delphinidin-glc absorbance at 520 nm (system 2). Identified compounds were quantified based on peak areas, applying an internal standard method for quercetin and an external standard method for delphinidin-glc.

Subsequently, HPLC-MS/MS analyses were performed to corroborate findings on quercetin and delphinidin-glc recoveries from cell lysates and to specifically search for potential delphinidin and delphinidin-glc metabolites phloroglucinol and gallic acid. While HPLC system 4 was used for delphinidin-glc, system 3 was applied for the remaining compounds. Single reaction monitoring was performed for specific detection of parent and product ions. Collision energies and characteristic ion transitions were chosen as follows:

Test compound	Parent ion (m/z)	Product ion (m/z)	Collision energy (eV)
Quercetin	301.0	151.2	30
Delphinidin-glc	465.1	302.9	20
Phloroglucinol	124.9	57.5	30
Gallic acid	169.0	125.1	20

Finally, HPLC-DAD-MS (system 4) analysis, generating a total ion chromatogram (TIC), was performed to address the identity of an unknown peak detected with HPLC-VIS in the delphinidin IMR-32 sample.

4.8.5 Interactions with the proteasome

4.8.5.1 Proteasome activity assay

For the proteasome activity assay, an aliquot of the cell suspension was drawn from the cell culture flask and centrifuged at 1,200 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended in the culturing medium. Effects of the test substances on the proteasomal chymotrypsin-like activity were determined using the chemiluminescent Proteasome Glo™ Cell-Based Assay according to the manufacturer's protocol (table 4-6).

Table 4-6 Proteasome assay protocol

Reagent	Volume (µl)	Incubation time (min)
HL-60 cells in culturing medium	100	
Test substance / DMSO	1	7
Assay buffer with substrate and luciferase	100	7

HL-60 cells in culturing medium were dispensed into 96-well microtiter plates at $1x10^4$ cells per well and flavonoid solutions were instantly added. An equivalent volume of DMSO was used as a negative control. Anthocyanins, anthocyanidins and procyanidins were dissolved and diluted in DMSO to yield final concentrations of 2, 5, 10, 50, and 100 µM in the assay. After incubation at room temperature for 7 min, assay buffer, containing the luminogenic proteasome substrate succinyl-leucine-leucine-valine-tyrosine-aminoluciferin and a recombinant firefly luciferase were added. Final substrate concentration amounted to 20 µM. Subsequent to further incubation at room temperature for 7 min, chemiluminescence, expressed as relative light units, was measured with a measuring time of 12 s for each well.

4.8.5.2 Data analysis

To quantify proteasome inhibition by test substances, the chymotrypsin-like activity of the proteasome was obtained using a coupled-enzyme system, with proteasome cleavage of the substrate releasing aminoluciferin and a luciferase reaction generating a chemiluminescence signal (figure 4-8).

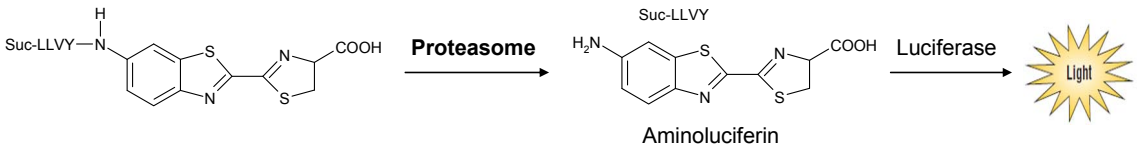

Figure 4-8 Proteasome catalyzed and subsequent reaction.

Chymotrypsin-like activity was calculated according to the following equation:

$$\%A = 100 \times \frac{A_I}{A_{DMSO}}$$

where %A is the percentage of the ChT-L proteasome activity remaining after treatment with test substances, A_I is the activity of the chymotrypsin site in the

presence of an inhibitor, and A_{DMSO} is the activity of the proteasome in the absence of inhibitors.

For each substance tested, mean values from three separate experiments performed in triplicate at five concentrations were analyzed with Prism v. 2.01 (GraphPad Software, CA, USA), using a non-linear regression model to determine the concentrations inhibiting 50% of the chymotrypsin-like proteasome activity. Mean IC_{50} values for anthocyanins and anthocyanidins were compared using an unpaired t-test. Statistical significance was set at p = 0.05.

4.8.6 Interactions with phospholipase A_2

4.8.6.1 Phospholipase A_2 activity assay

Studies on $sPLA_2$ enzyme inhibition were performed using a photometric assay based on the Ellman method [628]. Briefly, hydrolysis of the *sn*-2 ester bond of the substrate 1,2-bis(heptanoylthio)-glycerophosphocholine by PLA_2-V is followed by the exposure of free thiols. These trigger the conversion of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic (NTB) acid which is detected photometrically at 405 nm (figure 4-9).

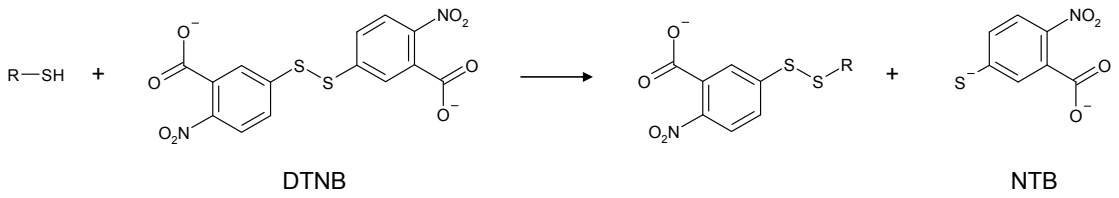

Figure 4-9 Ellman reaction: Colorless DTNB is converted to yellow 2-nitro-5-thiobenzoic acid in the presence of *sn*-2-thiols released upon PLA_2-V mediated substrate hydrolysis.

Prior to performing inhibition studies, linearity of product formation was investigated with regard to incubation time and various substrate, DTNB and enzyme concentrations to optimize assay conditions. Thereupon, the assay was carried out in an aqueous buffer solution (pH 7.5) containing KCl, $CaCl_2$, Tris and Triton-X 100 at final assay concentrations of 94 mM, 9 mM, 24 mM and 280 µM, respectively. Immediately before the assay was performed, substrate and PLA_2-V were resuspended in assay buffer and DTNB was dissolved in an aqueous solution of Tris-HCl (pH 8) with enzyme and DTNB yielding final concentrations of 100 ng/ml and 87 µM, respectively.

Assays were performed in 96-well microtiter plates at room temperature, containing DTNB, substrate solution plus the respective test substance. Thioetheramide phosphatidylcholine was used as a reference PLA_2 inhibitor and DMSO served as a

negative control. This solvent was shown to be inactive at the concentration used in the assay (1.7% v/v). Flavonoids were dissolved and diluted with DMSO. From the ethanolic solution of thioetheramide phosphatidylcholine, the solvent was evaporated under a stream of nitrogen and a DMSO solution was reconstituted and vortexed vigorously before further dilution. In terms of regression analysis, concentrations of anthocyanidins ranged from 0.3 to 160 µM, of anthocyanins from 3 to 700 µM, of procyanidin B2 from 2 to 1,500 µM, of catechin from 1 to 1,700 µM, of protocatechuic acid from 20 to 17,400 µM, and of thioetheramide phosphatidylcholine from 32 nM to 79 µM. In preliminary experiments, non-linear regression analysis was performed applying substrate at 0.3 mM.

For enzyme kinetic analyses, at least five substrate concentrations between 0.15 and 1.2 mM were used per concentration step and inhibition was measured at test compound concentrations ranging from 4 to 80 µM for anthocyanidins, and from 32 nM to 5 µM for thioetheramide phosphatidylcholine.

The phospholipase reaction was initiated by adding PLA_2-V, or assay buffer for control measurements. Absorption at 405 nm was recorded at intervals of 30 sec between 5 and 10 min thereafter.

Table 4-7 $sPLA_2$ assay protocol.

Reagent	Volume (µl)	Incubation time (min)
DTNB solution	10	
Substrate solution	200	
Test substance / DMSO	4	
Enzyme solution in buffer / buffer	16	5

4.8.6.2 Data analysis

For quantification of $sPLA_2$-V inhibition, enzyme activity was calculated using the absorption of 2-nitro-5-thiobenzoic acid at 405 nm. As the absorption is directly proportional to the amount of thiol released in the reaction with $sPLA_2$, the following equation applies:

$$\%A = 100 \times \frac{A_I}{A_{DMSO}}$$

where %A is the percentage of $sPLA_2$-V activity remaining after the exposure to test substances, A_I is the activity in the presence of an inhibitor, and A_{DMSO} is the enzyme activity in the absence of inhibitors (control). For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to eight

concentration steps, using a nonlinear regression model to determine the concentration inhibiting 50% of maximum $sPLA_2$ activity.

For enzyme kinetics analyses, experiments were performed at least twice in duplicate. Following normalization, the absorption was plotted against the incubation time. The resulting slope served as a measure of enzyme initial velocity (v) and was plotted against the respective substrate concentration [S] to obtain a substrate concentration versus velocity curve. Curves were then linearized by creating a reciprocal plot, or Lineweaver-Burk plot, which gave a family of intersecting lines for results of inhibition and control experiments. From this plot, the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated, while the line intersection point served to determine the mode of inhibition. The linear fit of the negative control was extrapolated to the point of x-axis intersection, with the negative abscissa intercept equalling $-1/K_m$ and the ordinate intercept equalling $1/V_{max}$.

Kinetic models considered for PLA_2 inhibition by anthocyanidins are outlined in figure 4-10. For calculation of further kinetic constants, i.e. the dissociation constant K_i, plus coefficients α and β for discrimination between complete and partial inhibition, secondary diagrams were generated plotting slope LB plot versus [I], 1/v versus [I] (Dixon plot), [S]/v versus [I] (Cornish-Bowden plot), 1/Δ y-axis intercept LB plot versus 1/[I] and 1/Δ slope LB plot versus 1/[I].

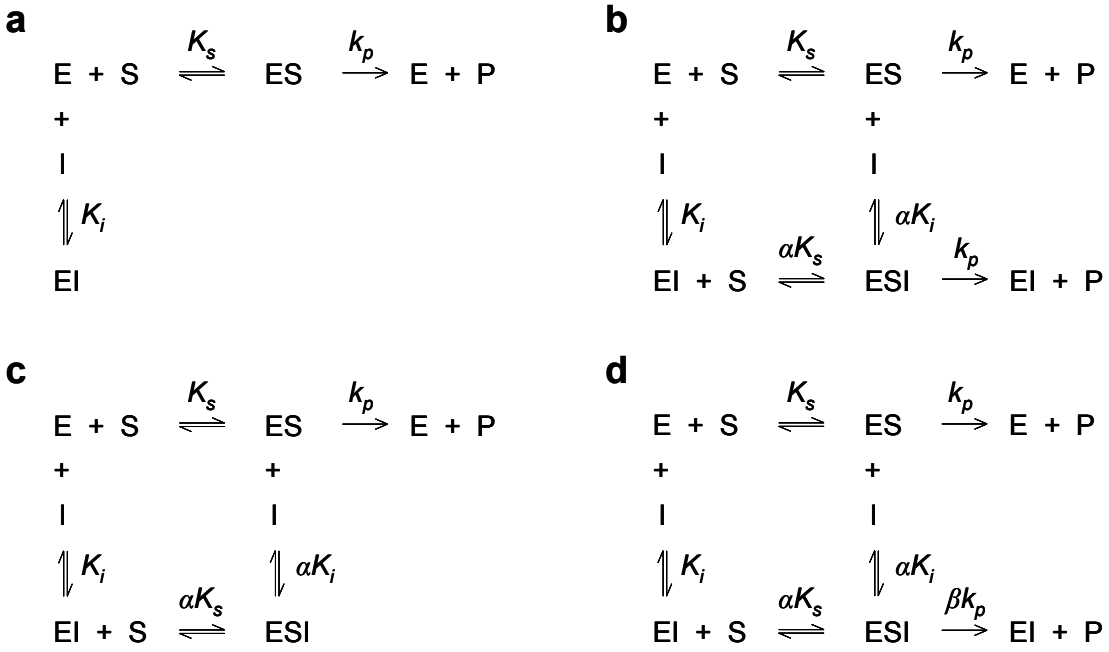

Figure 4-10 Kinetic models used to obtain K_i for competitive (a), partial competitive (b), mixed partial competitive and non-competitive (c) and mixed partial competitive and partial non-competitive (d) inhibition. Enzyme 'E' and substrate 'S' form the enzyme-substrate complex 'ES', enzyme and inhibitor 'I' form the enzyme-inhibitor complex 'EI'. K_s and K_i are the dissociation constants of 'ES' and 'EI', respectively. Dissociation constants αK_s and αK_i refer to the ternary complex 'ESI', coefficient β applies to functional 'ESI' when product formation is decreased [570].

For enzyme kinetics analysis, rapid equilibrium of the enzyme-substrate binding reaction was assumed, allowing to use K_m and K_s as equivalents [629].

4.8.7 Interactions with CYP3A4

4.8.7.1 CYP3A4 activity assay

Effects of test substances on CYP3A4 activity were determined using the P450-Glo™ Screening Systems, according to the manufacturer's protocol (table 4-8).

Table 4-8 CYP3A4 assay protocol.

Reagent	Volume (µl)	Incubation time (min)
Luciferin-free water	11.5	
Test substance / DMSO	1	
Reaction Mix	12.5	10
Tris-HCl	11.975	
Luciferin-PPXE	0.025	
CYP3A4 membranes	0.5	
NADPH regeneration system	25	20
Luciferin-free water	12	
Phosophate buffer	10	
Solution A	2.5	
Solution B	0.5	
Luciferin detection reagent	50	20

Briefly, a membrane preparation containing recombinant human CYP3A4, cytochrome P450 reductase and cytochrome b5 as an enhancer, was preincubated for 10 min at room temperature with the compound under study and luciferin-6' phenylpiperazinylyl ether (luciferin-PPXE), a luminogenic substrate. Assays were performed in 96-well microtiter plates in K_3PO_4 buffer. Ketoconazole and phenolic acids were dissolved in DMSO/H_2O (1:1), and the remaining compounds in DMSO. Anthocyanins, anthocyanidins, phenolic acids, and procyanidins B1 and B2 were diluted with DMSO/H_2O (1:1) to yield final concentrations ranging from 2 to 800 µM in the assay for tested polyphenols, and from 100 to 10,000 µM for phenolic acids. Ketoconazole was diluted to final concentrations ranging from 2 up to 200 nM.

$NADP^+$, glucose-6-phosphate, $MgCl_2$, and glucose-6-phosphate dehydrogenase served as a NADPH regeneration system and were added to start the enzymatic reaction. Final substance concentrations in the assay were 200 mM K_3PO_4, 24 mM Tris-HCl, 3.3 mM $MgCl_2$, 10 pmol/ml CYP3A4, 25 µM luciferin-PPXE, 1.3 mM $NADP^+$, 3.3 mM

glucose-6-phosphate and 0.4 U/ml glucose-6-phosphate dehydrogenase. Subsequent to further incubation at room temperature for 20 min, the reaction was stopped and a luminescent signal was initiated by adding a detection reagent, containing a firefly luciferase. After another 20 min, chemiluminescence values, displayed as RLUs, were recorded with a measuring time of 12 s for each well.

4.8.7.2 Data analysis

For quantification of CYP3A4 inhibition, enzyme activity was calculated using the light signal generated by oxidation of luciferin which, in turn, is produced by CYP3A4 dealkylation of the substrate luciferin-PPXE (figure 4-11).

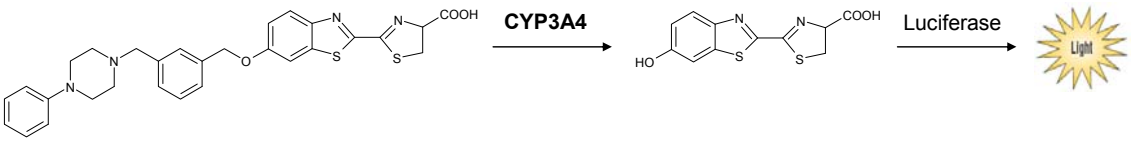

Figure 4-11 CYP3A4 catalyzed and subsequent reaction.

As the amount of light is directly proportional to the amount of luciferin released in the reaction with CYP3A4, the following equation applies:

$$\%A = 100 \times \frac{A_I}{A_{DMSO}}$$

where %*A* is the percentage of the CYP3A4 activity remaining after the exposure to test substances, A_I is the activity in the presence of an inhibitor, and A_{DMSO} is the enzyme activity in the absence of inhibitors (control).

For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to seven concentrations steps, using a nonlinear regression model to determine the concentration inhibiting 50% of maximum CYP3A4 activity.

To address a putative structural effect of anthocyanins' sugar component, substances were grouped by the number of sugar moieties, i.e., anthocyanins, anthocyanidin monoglycosides and anthocyanidin-diglucosides, and one-way ANOVA, followed by Bonferroni correction for multiple testing, was performed (Prism V 4.00, GraphPad Software, La Jolla, CA, USA, Statistica V 8.0, StatSoft, Tulsa, OK, USA). Moreover, two-way ANOVA was applied to investigate potential effects of B-ring substitution patterns and the presence or absence of sugar moieties. Statistical significance was set at $p = 0.05$.

4.8.8 Interactions with CYP2D6

4.8.8.1 CYP2D6 activity assay

The effects of test substances on CYP2D6 activity were determined using a validated and isoenzyme-specific P450-Glo™ Screening Systems according to the manufacturer's protocol (table 4-9).

Table 4-9 CYP2D6 assay protocol.

Reagent	Volume (µl)	Incubation time (min)
Luciferin-free water	11.5	
Test substance / DMSO	1	
Reaction Mix	12.5	10
Luciferin-free water	7.1	
Phosphate buffer	5	
Luciferin-ME EGE	0.15	
CYP2D6 membranes	0.25	
NADPH regeneration system	25	45
Luciferin-free water	22	
Solution A	2.5	
Solution B	0.5	
Luciferin detection reagent	50	20

At room temperature, a membrane preparation containing recombinant human CYP2D6 and cytochrome P450 reductase was preincubated for 10 min with the compound under study and the ethylene glycol ester of luciferin-6'-methylether (luciferin-ME EGE), a luminogenic substrate. The assay was perfomed in 96-well microtiter plates in KPO_4 buffer. Anthocyanins, anthocyanidins and procyanidin B2 were dissolved and diluted with DMSO to yield final concentrations ranging from 20 to 800 µM in the assay. Quinidine was diluted to final concentrations of 1, 10, 100 and 1,000 nM.

$NADP^+$, glucose-6-phosphate, magnesium chloride ($MgCl_2$) and glucose-6-phosphate dehydrogenase served as an NADPH regeneration system, and were added to start the enzymatic reaction. Final concentrations in the assay were 0.25 pmol CYP2D6, 100 mM KPO_4, 30 µM luciferin-ME EGE, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U/mL glucose-6-phosphate dehydrogenase and 50 µM sodium citrate. Subsequent to incubation at room temperature for 45 min, the reaction was stopped and a luminescent signal was initiated by adding a detection reagent that contained an esterase and a firefly luciferase. After another 20 min,

chemiluminescence values, displayed as RLUs, were recorded with a measuring time of 12 s for each well.

4.8.8.2 Data analysis

For quantification of CYP2D6 inhibition, enzyme activity was calculated using the light signal generated by oxidation of luciferin, which in turn is produced by CYP2D6 demethylation of the substrate luciferin-ME EGE (figure 4-12).

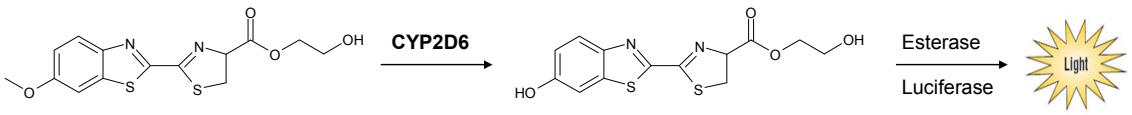

Figure 4-12 CYP2D6 catalyzed and subsequent reactions.

As the amount of light is directly proportional to the amount of luciferin released in the reaction with CYP2D6, the following equation applies:

$$\%A = 100 \times \frac{A_I}{A_{DMSO}}$$

where %*A* is the percentage of CYP2D6 activity remaining after the exposure to test substances, A_I is the activity in the presence of an inhibitor, and A_{DMSO} is the enzyme activity in the absence of inhibitors (control).

For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to five concentration steps, using a nonlinear regression model to determine the concentration inhibiting 50% of maximum CYP2D6 activity. To assess possible effects of structural features (anthocyanin sugar moiety and substitution pattern of the flavonoids' B-ring) on CYP2D6 inhibition, unpaired t-test and two-way ANOVA (Prism V 4.00, GraphPad Software, La Jolla, CA, USA, Statistica V 8.0, StatSoft, Tulsa, OK, USA) were performed. Bonferroni adjustment of p values was applied to correct for multiple testing. Statistical significance was set at $p = 0.05$.

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Appendix

Table A1 Overview of clinical studies on anthocyanin bioavailability.

Author	Dietary Administration (form and duration)	Anthocyanins
Cao and Prior [630]	25 mg **elderberry** extr	1500 mg: cya-glc, cya-sam
Murkovic et al. [489]	10 gel caps of 200 mg spray-dried elderberry j, breakfast	180 mg: cya-glc (66%), cya-sam (32%)
Murkovic et al. [631]	10 gel caps of 200 mg spray-dried elderberry j	180 mg
Cao et al. [90]	12 g elderberry extr, 500 ml w	720 mg: cya-3-sam, cya-3-glc
Milbury et al. [91]	12 g elderberry extr, 500 ml w	720 mg: cya-sam and cya-glc (92.5%)
Mülleder et al. [106]	11 g elderberry j concentr, w, w/wo 30 g sucr	1900 mg: mainly cya-glc and cya-sam
Wu et al. [632]	12 g elderberry extr, 500 ml w	720 mg: cya-sam and cya-glc (>90%),
Bitsch et al. [633]	150 ml elderberry j concentr, white rolls, cheese	3570 mg: cya-sam (63%), cya-glc (31%), cya-3,5-digly (6%)
Bitsch et al. [634]	30 ml elderberry extr, 107 ml w	147 mg: cya-sam (70%), cya-glc (30%)
Frank et al. [635]	150 ml elderberry j concentr	3569 mg: cya-sam (63%), cya-glc (31%), cya-diglc (6%)
Frank et al. [101]	30 / 200 ml elderberry extr, rolls	278 mg / 1852 mg: cya-sam (75%), cya-glc (25%), cya-diglc (0.1%)
Curtis et al. [415]	4 caps/d of elderberry extr, 12 weeks	500 mg/d: cya-sam-glc, cya-sam, cya-glc
Curtis et al. [415]	4 caps of elderberry extr, breakfast	500 mg: cya-sam-glc, cya-sam, cya-glc
Matsumoto et al. [488]	33 mg/kg bw **black currant** concentr, 150 ml w	3.58 mg (6.24 µmol)/kg bw: del-rut (47%), cya-rut (35%), del-glc (14%), cya-glc (4%)
Netzel et al. [636]	200 ml black currant j	153 mg: del-rut (51%), cya-rut (27%), del-glc (19%), cya-glc (3%)
Rechner et al. [412]	330 ml black currant j	1039 mg: del-rut (47%), cya-rut (34%), del-glc (10%), cya-glc (6%), unidentified (3%)
McGhie et al. [601]	300 ml black currant concentr	189 mg: cya-rut, del-rut, del-glc, cya-glc
Nielsen et al. [98]	4.4 g / 2.7 g black currant j concentr / 2.7 g with rice cake	1239 mg / 716 mg / 746: del-glc, del-rut, cya-rut, cya-glc
Bitsch et al. [634]	137 ml black currant j	145 mg: del-rut (41%), cya-rut (31%), del-glc (22%), cya-glc (6%)
Matsumoto et al. [100]	16.6 mg/kg bw black currant concentr, w, 1% phytic acid	4 mg/kg bw
Nakamura et al. [637]	253 mg black current extr, 100 ml w	59 mg (88 µmol): del-rut (41%), cya-rut (40%), del-glc (13%), cya-glc (6%)
Bub et al. [21]	500 ml **red grape** j, 150 g white rolls	117 mg malv-glc
Bub et al. [21]	500 ml dealcohol red wine, 150 g white rolls	58 mg malv-glc
Frank et al. [638]	400 ml red grape j, white rolls, 30 g cheese	284 mg: malv-glc (46%), peo-glc (29%), del-glc (18%), pet-glc (6%), cya-glc (1%)

Author	Dietary Administration (form and duration)	Anthocyanins
Bitsch et al. [639]	400 ml red grape j, white rolls, 30 g cheese	284 mg
Garcia-Alonso et al. [92]	12 g red wine extr, 125 g yoghurt, sugar, toast, butter	184 mg: malv-glc (49%), del-glc (24%), pet-glc (18%), peo-glc (7%),cya-glc (2%), (24% acyl)
Felgines et al. [104]	200 g **strawberries**, 15 g sugar, 60 g bread, 10 g butter	179 µmol: pel-glc, 3 minor
Carkeet et al. [103]	100 / 200 / 400 g strawberries, mixed with w	6.7 / 13.4 / 26.8 mg: pel-glc (88%), cya-glc (8%), pel-rut (4%)
Hollands et al. [107]	100 / 200 / 300 / 400 g strawberries, 72 g white toast, 10 g fat	57 / 114 / 171 / 228 mg: pel-glc (93%), minor cya-glc, pel-rut
Mullen et al. [93]	200 g strawberries w/wo 100 ml double cream	222 µmol: pel-glc, minor cya-glc, pel-3-O-(6''-rhaglc)
Mazza et al. [184]	100 g lowbush **blueberry** freeze-dried powder, 500 ml w, high-fat meal	1200 mg: gal, glc, ara of del, cya, pet, malv, plus acyl
Wu et al. [632]	189 g frozen lowbush blueberries, 315 ml w	690 mg
McGhie et al. [601]	blueberry extr, 300 ml w	439 mg: gal, glc, ara of del, cya, pet, malv
Kay et al. [88]	20 g **chokeberry** extr, 250 ml w	1321 mg cya-gly: cya-gal (68%), cya-ara (24%), cya-xyl (4%), cya-glc (4%)
Kay et al. [89]	7.1 g chokeberry extr in gel caps, 250 ml w	721 mg cya-gly: cya-gal (68%), cya-ara (24%), cya-xyl (4%), cya-glc (4%)
McGhie et al. [601]	**boysenberry** extr, 300 ml w	345 mg: cya-glc, cya-soph, cya-3-O-2G-glcrut, cya-rut
Cooney et al. [640]	2 x 30 g boysenberry extr, 300 ml w, 2 h interval	cya-3-soph, cya-glc, little cya-3-O-2G-glcrut, cya-3-rut
Lehtonen et al. [641]	300 g **lingonberries**, vanilla yoghurt	193 mg: cya-gal (92%), cya-glc (8%)
Nurmi et al. [99]	50 g bilberry-lingonberry purée, w/wo 50 g oat cereals	650 mg (1435 µmol)
Felgines et al. [20]	200 g **blackberries**, 15 g sugar, 60 g bread, 10 g butter	960 µmol: cya-glc (89.%),cya-pent (6%), acyl cya-glc (5%)
Hassimotto et al. [97]	blackberries homogenized, 200 ml w / defatted milk	400 mg/50 kg bw: cya-glc (91%)
Stoner et al. [413]	45 g/d freeze-dried **black raspberry** powder, w, 7 d	1440 mg/d: cya-rut (67%), cya-xylrut (18%), cya-glc (9%), cya-sam (6%)
Tian et al. [414]	45 g/d freeze-dried black raspberry powder, w, 7 d	2691 mg/d: cya-rut (61%), cya-xylrut (26%), cya-glc (8%), cya-sam (5%)
González-Barrio et al. [87]	300 g **raspberries** homogenized	204 µmol: cya-soph (55%), cya-(2''-glc)-rut (22%), cya-glc (13%)
Riso et al. [96]	600 ml/d **blood orange** j, 21 d	28.2 mg/d: cya-glc (74%), cya-(-6''-mal)-glc (26%)
Vitaglione et al. [24]	1 l blood orange j	72 mg: cya-glc (60%), cya-(6''-mal)-glc (39%), pel-glc (1%)
Ohnishi et al. [642]	200 ml **cranberry** j	651 µg: peo-ara (35%), peo-gal (27%), cya-gal (20%), cya-ara (16%), peo-glc (2%)
Mertens-Talcott et al. [102]	7 ml/kg bw **acai** pulp, clarified acai j	304 mg/kg bw (pulp), 166 mg/kg bw (j)

Author	Dietary Administration (form and duration)	Anthocyanins
Frank et al. [643]	10 g **Hibiscus** sabdariffa L. extr, 150 ml w, white rolls	147 mg: cya-sam (42%), del-sam (56%),del-glc (2%), cya-glc (0.1%)
Miyazawa et al. [625]	1.6 g spray dried **fruit** j (elderberry, black currant), 20 ml w	2.95 mg/kg bw: cya-glc (92%), cya-diglc (8%)
Kurilich et al. [95]	250 g raw / 250 g cooked / 500 g cooked **purple carrots**	250 g raw: 416 mg (463 μM) / 250 g cooked: 321 mg (357 μM), cya-gly, acyl (86%)
Charron et al. [94]	50 / 150 / 250 ml purple carrot j	65 / 194 / 323 mg, acyl (76%)
Harada et al. [644]	125 ml **purple sweet potato** extr beverage	311 mg: cya-3-soph-5-glc and peo-3-soph-5-glc, 8 acyl
Kano et al. [645]	375 ml purple sweet potato extr beverage	933 mg, 8 acyl
Mullen et al. [646]	270 g fried **red onions**	75 μmol
Charron et al. [105]	100 / 200 / 300 g steamed **red cabbage**, salt, margarine	138 / 277 / 415 μmol, acyl (79%)

Author	Compounds identified in plasma/serum	Plasma/serum t_{max}	Plasma/serum c_{max}
Cao and Prior [630]	original glycosylated forms	N/D	30 min: >100 ng/ml
Murkovic et al. [489]	original glycosylated forms	3 h	≈1.4 ng/ml
Murkovic et al. [631]	original glycosylated forms	1 h	35 ng/ml
Cao et al. [90]	original glycosylated forms, low aglycone, no glucuron or sulfates	1.2 h	97 nM
Milbury et al. [91]	original gycosylated forms, aglycone, no glucuron or sulfates	1.2 h	97 nM
Bitsch et al. [634]	bioavailability: cya-glc>cya-sam	1.5 h	15.6 ng/ml
Frank et al. [101]	linear absorption with dose	1.5 h / 1.25 h	30 ng/ml / 220 ng/ml
Curtis et al. [415]	no anth or glucuron, sulfates (after overnight fast)	N/D	N/D
Curtis et al. [415]	original glycosylated forms, glucuron and sulfates	≥3 h	15 ng/ml, 20 ng/ml glucuron and sulfates
Matsumoto et al. [488]	original glycosylated forms	1.75 h del-rut, 1.5 cya-rut, 1.5 del-glc, 1.25 cya-glc	121 nM
Rechner et al. [412]	original glycosylated forms	1 h	85 nM
Nielsen et al. [98]	increased with dose, not influenced by rice cake, but delayed absorption, bioavailability: rut>glc	0.75 / 0.75 / 1.5 h	≈50 ng/ml / ≈15 ng/ml / ≈30 ng/ml
Bitsch et al. [634]	bioavailability: cya-glc>del-glc>cya-rut=del-rut	1 h	2.9 ng/ml
Matsumoto et al. [100]	phytic acid increased anth concentr, enhanced absorption	1 h (2 h wo phytic acid)	5.2 ng/ml (0.46 ng/ml wo phytic acid)
Nakamura et al. [637]	original glycosylated forms, t_{max} rut>glc	0.75 h del-glc, cya-glc, 1.25 h del-rut, cya-rut	6.9 nM
Bub et al. [21]	no aglycone, no sulfate, no glucuron	2 h	2.8 nM, 1.5 ng/ml
Bub et al. [21]	no aglycone, no sulfate, no glucuron, sugar may delay absorption	1.5 h	1.7 nM, 0.9 ng/ml
Frank et al. [638]	N/D	0.5 h	100 ng/ml
Bitsch et al. [639]	original glycosylated forms	0.5 h	100 ng/ml
Garcia-Alonso et al. [92]	malv-glc (53%), peo-glucuron, malv-glucuron, little del-glc, pet-glc, no cya-glc	1.4 h peo-glc, 1.8 h malv-glc, 2.4 h peo-glucuron, 2.6 malv-glucuron	7.9 nM
Mullen et al. [93]	main pel-glucuron, 3 minor pel-monoglucuron, pel-glc, decreased and delayed by cream	wo 1.1 h, w 2.4 h	wo 274 nM, w 227 nM pel-glucuron

Author	Compounds identified in plasma/serum	Plasma/serum t_{max}	Plasma/serum c_{max}
Mazza et al. [184]	original glycosylated forms	4 h	13.1 ng/ml
Wu et al. 2002 [632]	below detection limit	N/D	N/D
Kay et al. [88]	glucuron, methlylated deriv of cya-gal and cya-glucuron	2 h	351 nM
Kay et al. [89]	glucuruon (63%), methlyated deriv of cya-gly	2.8 h	96 nM
Nurmi et al. [99]	cereal delayed a absorption, no difference in c_{max}	w: 3 h, wo: 1.5 h	w: 149 nM, wo: 138 nM
Hassimotto et al. [97]	milk prevented absorption (4 h)	in w: 0.5-1 h	in w: 140 μM
Stoner et al. [413]	original glycosylated forms, no glucuron, daily dosing did not enhance absorption	1.1 h cya-glc, 1.6 h cya-rut, 2.2 h cya-sam, 2.6 h cya-xylrut (day1, 7)	33.7 ng/ml (day1, 7)
González-Barrio et al. [87]	below detection limit	N/D	N/D
Riso et al. [96]	overnight fast: 8.33 nM cya-glc, no aglycone, no cya-mal-glc	N/D	N/D
Vitaglione et al. [24]	cya-glc, protocatechuic acid (44% of ingested), no cya-mal-glc	0.5 h cya-glc, 2 h protocatechuic acid	1.9 nM cya-glc, 492 nM protocatechuic acid
Mertens-Talcott et al. [102]	higher dose caused lower bioavailability	2.2 h (pulp), 2 h (j)	2321 ng/ml (pulp), 1138 ng/ml (j)
Frank et al. [643]	cya-sam>del-sam	1.5 h	3.4 ng/ml
Miyazawa et al. [625]	1 h: 29 nM cya-glc, minor cya-diglc, no aglycone, no glucuron or sulfate or methyl forms	N/D	N/D
Kurilich et al. [95]	nonacyl>acyl, for nonacyl increased by cooking, reduced with dose	2 h	5.8 / 5.3 / 5.0 nM
Charron et al. [94]	nonacyl>acyl, reduced with dose	2 / 1 / 2 h	2.5 nM / 6.6 nM / 9.6 nM
Harada et al. [644]	original glycosylated, acylated forms	1-1.5 h	≈2.4 nM
Kano et al. [645]	cya-cafsoph-glc, peo-cafsoph-glc	3 h	4.48 nM peo-cafsoph-glc
Mullen et al. [646]	below detection limit	N/D	N/D

Author	Compounds identified in urine	Urinary t_{max}	Urinary excretion	Urinary excretion (% of ingested)
Cao et al. [90]	unknown metabolites	N/D	397 µg (1-24 h)	0.055
Milbury et al. [91]	original glycosylated forms (cya-sam and cya-glc), aglycone	N/D	397 µg (0-24 h)	0.055
Mülleder et al. [106]	cya-glc and cya-sam, unidentified metabolites, sucr reduced and delayed urinary excretion	wo sucr: 1-2 h, w sucr: 3 h	N/D	0.003-0.012
Wu et al. 2002 [632]	cya-sam, minor: peo-glc, peo-sam, peo-monoglucuron, cya-glc-monoglucuron: methylated, glucuron	N/D	554 µg (0-4 h)	0.077
Bitsch et al. 2004 [633]	glycosides, minor glucuron	1 h	1760 µg a, 116 µg cya-glucuron	0.05 anth, 0.003 glucuron
Bitsch et al. [634]	cya-sam>cya-glc	1.5 h / 1 h	548 µg	0.37 (0.38 cya-sam, 0.25 cya-glc)
Frank et al. [635]	cya-diglc>cya-glc=cya-sam	1 h	2055 µg (0-24 h)	0.06 (0.16 cya-diglc, 0.06 cya-glc, 0.05 cya-sam)
Frank et al. [101]	traces of metabolites, higher % excretion with smaller dose	1.5 h / 1 h	1070 µg / 5030 µg (0-7 h)	0.39 / 0.27
Matsumoto et al. [488]	all 4 recovered as intact glycosides, rut (cya, del)>glc	N/D	143 nmol (82 µg) (0-8 h)	0.11 del-rut, 0.098 cya-rut, 0.066 del-glc, 0.06 cya-glc
Netzel et al. [636]	cya-rut>del-glc>del-rut>cya-glc	2 h	53 µg (0-5 h)	0.026 del-rut, 0.05 cya-rut, 0.035 del-glc, 0.02 cya-glc
Rechner et al. [412]	rut (cya, del)>glc	1 h	420 µg (32 h)	0.046 del-rut, 0.045 cya-rut, 0.039 del-glc, 0.032 cya-glc
McGhie et al. [601]	rut (cya, del)>glc	2 h	120 µg	0.064 (0.067 del-rut, 0.063 cya-rut, 0.042 del-glc, 0.04 cya-glc)
Nielsen et al. [98]	higher % excretion with higher dose, not influenced by rice cake	N/D	N/D	0.072 / 0.048 / 0.045 (0-4 h)
Bitsch et al. [634]	cya-glc>cya-rut=del-rut>del-glc	1.5 h	63.5 µg	0.043 (0.053 cya-glc, 0.048 cya-rut, 0.047 del-rut, 0.025 del-glc)
Matsumoto et al. [100]	phytic acid increased anth excretion	0-2 h	356 µg (79 µg control)	N/D
Bub et al. [21]	no aglycone, no sulfate, no glucuron	N/D	27 µg (1-6 h)	0.023
Bub et al. [21]	no aglycone, no sulfate, no glucuron	N/D	13.3 µg (1-6 h)	0.023
Frank et al. [638]	pet-glc>peo-glc>del-glc>malv-glc>cya-glc	0.5 h	654 µg (0-7 h)	0.23 (0.32 pet-glc, 0.29 peo-glc, 0.2 del-glc, 0.18 malv-glc, 0.09 cya-glc)
Bitsch et al. [639]	only glycosides, pet-glc, peo-glc>cya-glc	0.5 h	654 µg	0.23

Author	Compounds identified in urine	Urinary t_{max}	Urinary excretion	Urinary excretion (% of ingested)
Garcia-Alonso et al. [92]	peo-glucuron, malv-glucuron, 72% glc, 28% glucuron,	0-3 h, malv-glucuron 3-6 h	74 µg	0.05 (0.03 pet-glc, 0.06 del-glc, 0.06 peo-glc and -glucuron, 0.06 malv-glc and glucuron)
Felgines et al. [104]	pel-glc and 5 metabolites (3 pel-monoglucuron, pel sulfate, pel aglycone), pel-glucuron (90%)>pel sulfate>pel-glc>pel	0-2 h, main glucuron 2-4 h	3226 nmol (0-24 h)	1.8 (1.61 pel-glucuron, 0.066 pel-glc, 0.075 pel sulfates, 0.044 pel)
Carkeet et al. [103]	pel-glc and 3 pel-glucuron (>90%)	2 h	321 nmol / 676 nmol / 1163 nmol (0-24 h)	2.1 / 2.2 / 1.9
Hollands et al. [107]	pel-glc, 3 pel-monoglucuron, pel monosulfate, pel aglycone	N/D	N/D	1.71 (1.84 / 1.77 / 1.67 / 1.76)
Mullen et al. [93]	one main pel-glurcuron, minor 3 other pel-glucuron, pel-glc, pel and pel sulfate, % excretion not influenced by cream, but delayed	wo 0-2 h, w 2-5 h	wo 1672 nmol, w 2217 nmol (0-24 h)	without 0.75, with 1
Wu et al. [632]	original glycosylated forms, ara of del, pet, malv at lower concentr compared to extract	2-4 h	23 µg (0-6 h)	0.004
McGhie et al. [601]	glc, gal>ara	2 h	87 µg	0.02 (0.03 del-glc, 0.027 pet-gal, 0.026 malv-gal)
Kay et al. [88]	glucuron, methlylated, oxidized deriv of cya-gal and cya-glucuron,	N/D	N/D	N/D
Kay et al. [89]	glucuron, methlyated deriv of cya-gly, 32.5% parent comp, 67.5% metabolites	3.7 h	1072 µg (0-24h)	0.15 (0.048 parent comp)
McGhie et al. [601]	cya glcrut≈soph>rut≈glc	2 h	99 µg	0.029 (0.03 cya-glcrut, 0.026 cya-soph)
Cooney et al. [640]	all comp recovered, metabolites: 2 peo-glucuron, cya-glucuron, pel-glucuron, methylated, OH-loss	N/D	N/D	N/D
Lehtonen et al. [641]	cya-gal (46.7%), peo-gal (=methyl, 30.7%), cya-glucuron (22.6%), trace: cya-glc, cya-ara, peo-ara	4-8 h	N/D	N/D
Nurmi et al. [99]	N/D	N/D	N/D	<0.01
Felgines et al. [20]	peo-glucuron>cya-glucuron (64%)>cya-glc>cya>peo-glc>peo, cya-diglucuron, methylated, deglycosylated	2-4 h	1537 nmol (0-24 h)	0.16 (0.063 peo-glucuron, 0.040 cya-glucuron, 0.023 cya-glc, 0.012 cya, 0.0098 peo-glc, 0.0045 peo)
Hassimotto et al. [97]	N/D	2-4 h	N/D	N/D
Stoner et al. [413]	no glucuron, cya-sam>cya-xylrut>cya-rut>cya-glc, predose concentr on day 7: 24 µg	0-4 h	492 µg (day 7)	0.058 cya-sam, 0.046 cya-xylrut, 0.032 cya-rut, 0.016 cya-glc (rel to daily dose)

Author	Compounds identified in urine	Urinary t_{max}	Urinary excretion	Urinary excretion (% of ingested)
Tian et al. [414]	all 4 recovered, 2 methyl deriv of cya-xyl-rut, 1 of cya-rut, traces: cya-sam, cya-glc, no sulfates, no glucuron, cya-xylrut>cya-rut	4-8 h	492 µg (day 7)	N/D
González-Barrio et al. [87]	3 principal recovered, cya-soph, cya-(2''-glc)-rut, cya-glc	0-4 h	78 nmol (0-48 h)	0.04
Vitaglione et al. [24]	cya-glc, cya-malonyl-glc, glucuron and methyl metabolites, no protocatechuic acid	2-12 h	1850 nmol	1.2 (0.02 cya-glc and cya-malonyl-glc, 0.8 cya-glucuron, 0.3 cya-methyl-glucuron)
Ohnishi et al. [642]	6 anth, no glucuron, high urinary excretion, peo-gal=peo-glc>cya-gal=cya-ara>peo-ara>cya-glc	3-6 h	74 nmol	5 (11 peo-gal, 11 peo-glc, 3.7 cya-gal, 3.6 cya-ara, 2 peo-ara, 1.4 cya-glc)
Frank et al. [643]	del-sam>cya-sam	1.5 h	27.2 µg (0-7 h)	0.018 (0.016 cya-sam, 0.021 del-sam)
Kurilich et al. [95]	4 intact, cooking increased nonacyl, % excretion decreased with higher dose, nonacyl>acyl	4 h	140 nmol / 135 nmol / 146 nmol	0.03 / 0.038 / 0.02 (0.013 / 0.014 / 0.008 acyl, 0.14 / 0.19 / 0.1 nonacyl)
Harada et al. [644]	cya-cafsoph-glc, peo-cafsoph-glc	N/D	≈8 nmol	0.01-0.03
Kano et al. [645]	cya-cafsoph-glc, peo-cafsoph-glc	2-6 h	N/D	0.01
Mullen et al. [646]	below detection limit	N/D	N/D	N/D
Charron et al. [105]	3 nonacyl, 8 acyl, 4 glucuron and methylated metabolites, nonacyl>acyl, an of acyl, % excretion decreased with higher dose	2 to 4 h	115 / 138 / 178 nmol	0.073 / 0.042 / 0.036 (0.041 / 0.023 / 0.02 acyl, 0.176 / 0.105 / 0.085 nonacyl)

Abbreviations: acyl, acylated; anth, anthocyanin; ara, arabinoside; bw, body weight; caf, caffeoyl; comp, compound; concentr, concentration, concentrate; cya, cyanidin; d, day; dealcohol, dealcoholized; del, delphinidin; deriv, derivate; extr, extract; gal, galactoside; gel caps, gelatinous capsules; glc, glucoside; glucuron, glucuronide; gly, glycoside; j, juice; malv, malvidin; N/D, not determined, pel, pelargonidin; pent, pentose; peo, peonidin; pet, petunidin; rel, relative; rha, rhamnoside; rut, rutinoside; sam, sambubioside; soph, sophoroside; sucr, sucrose; w, water; w/wo, with/without; xyl, xyloside.
Single doses were administered unless indicated otherwise.