Why do we expect flavonoids to function as antioxidants *in vivo*?

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FLAVONOIDS:
FOCUS OF MUCH CURRENT NUTRITIONAL
AND THERAPEUTIC INTEREST

- CARDIOPROTECTION
  Role for flavonoid-rich dietary components in reduc
tion in risk of cardiovascular disease

- NEUROPROTECTION
  Anthocyanin-rich fruit associated with protes
tion against age-related decline in cognitive function

- CHEMOPREVENTION
Flavonoids: naturally occurring low molecular wt phenols consisting of 2 benzene rings linked via a heterocyclic pyrone or pyran ring -> patterns and substitutions comprising the sub-classes:

- **Anthocyanin** - berries
- **Flavanone** - citrus
- **Flavanol** - red wine, teas, chocolate, fruit
- **Flavonol** - fruit, vegetables
- **Hydroxycinnamates** - most fruit & some vegetables
Flavonol
*e.g.* quercetin
onion, cranberry, red apple
many fruit and vegetables

Flavanol
*e.g.* epicatechin
red wine, green tea,
as procyanidins in apple, chocolate

Flavanone
*e.g.* hesperetin
*Citrus* fruit, orange

Hydroxycinnamate
*e.g.* caffeic acid
most fruit especially tomato, apple
some vegetables e.g. eggplant, grains

Anthocyanidin
*e.g.* cyanidin
major constituents of dark
red fruit berries e.g. raspberries

Flavanone
SMALL DIFFERENCES IN STRUCTURE → LARGE CHANGES IN BIOLOGICAL ACTIVITIES

Number and specific positions of OH groups / nature of substitutions determine whether flavonoids function as:

- antioxidant, anti-inflammatory, cytotoxic or antimutagenic agents in vitro or in vivo.

- Antioxidant/pro-oxidant activities
- Enzyme induction / inhibition
- Cell proliferation / growth inhibition
- Lipophilicity / polarity - cellular access
PROTECTIVE PROPERTIES OF FLAVONOIDS AGAINST OXIDATIVE STRESS ARE STRUCTURE-DEPENDENT

• Scavengers of reactive oxygen species - H-donating abilities
• Transition metal chelators – catechol requirement?
• Scavengers of reactive nitrogen species nitric oxide, peroxynitrite etc – nitration or oxidation?
• Non-antioxidant mechanisms - modulation of signaling pathways, gene expression
STRUCTURAL REQUIREMENTS FOR H-DONATING ANTIOXIDANT ACTIVITY:

*ortho*-dihydroxy substitution in B-ring
2,3-unsaturation in C-ring
4-carbonyl group

Bors *et al.* 1990; Rice-Evans *et al.* 1996;

QUERCETIN
<table>
<thead>
<tr>
<th>CATECHOLS</th>
<th>Reduction potentials E&lt;sub&gt;7&lt;/sub&gt;</th>
<th>Antioxidant activity TEAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>0.33</td>
<td>4.7</td>
</tr>
<tr>
<td>epicatechisin</td>
<td>0.57</td>
<td>2.4</td>
</tr>
<tr>
<td>MONOHYDROXY B-RING</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kaempferol</td>
<td>0.75</td>
<td>1.3</td>
</tr>
<tr>
<td>hesperetin</td>
<td>0.72</td>
<td>0.9</td>
</tr>
<tr>
<td>ALKYLPEROXYL RADICAL</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>VITAMIN C</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Jovanovic et al. 1998; Rice-Evans et al. 1996
STRUCTURAL DETERMINANTS OF CYTOTOXICITY

- Ease of oxidation – catechol vs monophenolic

- Lipophilicity
OXIDATION OF QUERCETIN

Damage through adduct formation with proteins, GSH, RNA and DNA

Quercetin

Quercetin-7-quinone methide

Quercetin-5-quinone methide

α-quinone
STRUCTURAL DEPENDENCE OF PEROXIDATIVE METABOLISM OF FLAVONOIDS – *monophenolic B-ring*

\[ \text{FlavOH} + \text{ferryl radical} \rightarrow \text{FlavO}^\bullet \quad \text{Phenoxyl radical} \]

\[ \text{FlavO}^\bullet + \text{GSH} \rightarrow \text{GS}^\bullet \quad \text{Thiyl radical} \]

\[ \text{GS}^\bullet + \text{O}_2 \rightarrow \text{Reactive oxygen species} \rightarrow \text{GSSG} \]

*Galati et al. 2002*
WHAT’S HAPPENING IN VIVO?
STRUCTURAL CHANGES ON ABSORPTION

Influence of conjugation and metabolism on structural parameters governing biological properties
MAJOR METABOLIZING ENZYMES:
small intestine / liver / colon

- Glucosidases
- UDP-glucuronosyl transferases
- Catechol-O-methyl transferases
- Sulfotransferases
- Hydrolases
- Esterases
- Cytochrome P450s

OTHERS:
- Glutathione-S transferases
- Quinone reductases
Absorption and Biotransformation of Dietary Flavonoids *In Vivo*

- **Stomach**
- **Small Intestine**
  - jejunum
  - ileum
- **Colon**
- **Gut microflora**
- **Oligomeric Flavonoids** → **Oligomers cleaved** → **Monomeric units**
- **Phase I and II metabolism**
- **Portal vein**
- **Liver**
  - **Further metabolism**
  - O-methylated
  - Sulphates
  - glucuronides
  - glucuronides
- **Kidney**
  - **Renal excretion of glucuronides**
- **Urine**
- **SKIN AND BRAIN cells**
- **Renal excretion** of glucuronides

**Flavonoid** → **Phenolic acids**

*Gut microflora*
POTENTIAL MOLECULAR SITES OF METABOLIC MODIFICATION

- glucuronidation
- sulphation
- methylation
- oxidation
- cleavage
EFFECTS OF METABOLISM ON FLAVONOID STRUCTURES – IMPLICATIONS FOR BIOLOGICAL PROPERTIES

epicatechin

3'-O-methyl-epicatechin

epicatechin-7-β-D-glucuronide

4'-O-methyl-epicatechin-7-β-D-glucuronide
STRUCTURAL FACTORS INFLUENCING INTRACELLULAR ANTIOXIDANT PROPERTIES

- Reduction potentials of resulting conjugates
- Cellular access and partition coefficients
- Intracellular/extracellular metabolism and structural modifications
FLAVONOIDS CAN BE EXTENSIVELY METABOLISED BY cytP450s
-> metabolites with modified biological activities–human liver microsomes

Breinholt et al. 2002
STRUCTURAL CONSEQUENCES OF INTRACELLULAR METABOLISM

quercetin

4'-O-methyl quercetin

demethylation

3'-O-methyl quercetin

GSH, cys, protein thiol

glucuronide/glucoside

GSH, cys, protein thiol

??

GLUTATHIONE
Quercetin

RT: 55.43

Quercetin

3´-O-Me-quercetin

Spencer et al. 2002
COLONIC BIOTRANSFORMATION

WHAT’S HAPPENING IN THE COLON?

Majority of ingested flavonoids undergo colonic metabolism
small intestine
bacterial numbers:
c.a. $10^4$-$10^6$/ml contents
e.g. lactobacilli,
Gram positive cocci

stomach
bacterial numbers:
c.a. $10^3$/ml contents
e.g. Helicobacter pylori

colon
bacterial numbers:
c.a. $10^{12}$/g contents
bacteroides, bifidobacteria, clostridia, peptostreptococci, fusobacteria, lactobacilli, enterobacteria, enterococci, eubacteria, methanogens, sulphate reducers etc
Pathway of the colonic degradation of rutin - implications for properties of in vivo metabolites

Deglycosylation

Ring fission, water elimination, dehydroxylation

Further degradation

Protocatechuic acid

β-Oxidation + glycination

3-hydroxyhippuric acid

Absorption from the colon

Rutin

Deglycosylation

3,4-dihydroxyphenylacetic acid

Dehydroxylation

3-hydroxyphenylacetic acid

Quercetin

β-Oxidation + glycination

3-hydroxyhippuric acid
MAJOR COLONIC METABOLITES

- 3,4-dihydroxyphenyl acetic acid
- 3-(3-hydroxyphenyl)propionic acid
- 3-(4-hydroxyphenyl)propionic acid
- Hydroxybenzoates
SO DO WE EXPECT FLAVONOID TO
BE ANTIOXIDANTS IN VIVO?

IT DEPENDS:

- on what we mean by ‘antioxidation’

- on the extent and structural consequences of conjugation and metabolism
BIOAVAILABILITY AND METABOLISM OF FLAVONOIDS

• Less bioavailable than ascorbate and tocopherols

• MODIFIED by metabolism on absorption

• Less extensively absorbed and circulating levels *in vivo* much lower
**PLASMA LEVELS OF FLAVONOID CONJUGATES**

<table>
<thead>
<tr>
<th>Flavan-3-ol:</th>
<th>100 nM</th>
<th>METHYL + SULPHATE +GLUCURONIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wine catechins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procyanidin:</td>
<td>4 uM; 0.26 uM;</td>
<td>EPICATECHIN SULPHATE + GLUCURONIDE</td>
</tr>
<tr>
<td>Chocolate/cocoa</td>
<td>0.7 uM</td>
<td></td>
</tr>
<tr>
<td>Flavanone – grapefruit/orange</td>
<td>&lt; 4 uM</td>
<td>NARINGENIN/HESPERETIN GLUCURONIDE</td>
</tr>
<tr>
<td>Anthocyanin – berry juices</td>
<td>100 nM; 147 nM</td>
<td>ANTHOCYANIN GLYCOSIDES</td>
</tr>
</tbody>
</table>

Donovan et al., Keen et al., Baba et al., Ameer et al, Miyazawa et al.
IN VIVO METABOLITE FORMS VERSUS CELLULAR OXIDATIVE STRESS

Increase in Absorbance (405 nm)

Control  H₂O₂ (50 µM)  EC  MeEC  EC Gluc

All 30 µM

H₂O₂ (50 µM)

METHYLATED METABOLITE
Lower H-donating potential – *modified catechol group*
Similar protective effects against oxidative stress-induced cell death

GLUCURONIDE METABOLITE
Marginally lower H-donating potential
No protective effects against oxidative stress-induced cell death - *inaccessibility or substituted A-ring?* SPENCER et al. 2001
PROTECTION OF NEURONS FROM OXIDATIVE STRESS-INDUCED CELL DEATH BY EPICATECHIN

I Control neurons
II Neurons exposed to oxidative stress
III Control neurons treated with epicatechin
IV Neurons pretreated with epicatechin prior to oxidative stress

Schroeter et al. 2000
CONCLUSIONS:

- BIOACTIVITY OF FLAVONOIDs *in vivo* MAY NOT DEPEND ON THEIR ACTIVITIES AS DIRECT SCAVENGERS OF REACTIVE OXYGEN OR NITROGEN SPECIES *PER SE*

- BUT RATHER ON THE INFLUENCE OF THEIR *IN VIVO* FORMS ON THE MODULATION OF ENZYME / PROTEIN FUNCTIONS, INTRACELLULAR CELL SIGNALLING AND RECEPTOR ACTIVITIES