The use of flavonoid aglycones in in vitro systems to test biological activities: based on bioavailability data, is this a valid approach?

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Abstract
The majority of in vitro assays on biological activities of flavonoids have used the aglycone form as the test compound. This form is readily available from commercial sources and comparable approaches have been used for testing efficacy of drugs. This paper presents the hypothesis that aglycones are only transiently present in vivo at significant concentrations at specific sites. The pathway of metabolism of flavonoids in mammals in vivo, focusing on aglycone formation, is examined to facilitate better design in the future of in vitro cell culture experiments. In vitro experiments using flavonoids and cultured cells require careful consideration of absorption and bioavailability for their appropriate interpretation.

Introduction
Flavonoids and isoflavonoids such as quercetin, kaempferol, genistein, daidzein, naringenin and hesperidin occur in plants and food as glycosides. Flavanols such as catechins and the oligomeric proanthocyanidins are unglycosylated and occur naturally as the aglycone form. A summary of the flavonoid metabolites and their formation as discussed in this paper is presented in Figure 1.

The food
Glycosylated flavonoids are stable to cooking, microwaving or boiling (Price et al., 1997), but fermentation leads to partial deglycosylation (Kishida et al., 2000). Thus aglycones of quercetin are found in red wine (Gaspar et al., 1993) and aglycones of isoflavones are found in fermented soy products (Kishida et al., 2000). Flavanols and proanthocyanidins are found naturally as free aglycone forms, and these are clearly consumed in food in an unconjugated form. The absorption of flavonoids is also affected by the vehicle in which they are presented to the body i.e., food matrix, dissolution and nature of solvent. This is especially significant when aglycones of compounds usually present in the food as glycosides are administered. The amount of quercetin in plasma, for example, is dramatically affected by the nature of the solvent used to administer the compound (Azuma et al., 2002). On the other hand, the presence of alcohol in red wine did not affect the plasma concentrations in humans of catechin, an unglycosylated flavonoid (Donovan et al., 2002a).

Stomach modifications
Flavonoid glycosides and flavanol monomers are generally stable to stomach pH and secreted gastric enzymes. Also, although proanthocyanidins break down in vitro at pH 2 over several hours to monomeric flavanols and unidentified compounds (Spencer et al., 2000), they are stable in the stomach in humans in vivo (Rios et al., 2002), but may not be absorbed into plasma in an intact form (Donovan et al., 2002b). Quercetin and isoflavones consumed as aglycones are absorbed in rat stomach (Piskula, 2000), but glyco-
sides (of quercetin) are not (Crespy et al., 2002). However, the capacity of the stomach to absorb compounds is limited compared to the small intestine; the absorptive surface in stomach is 4000 times less in humans (0.05 m² compared to 200 m² in small intestine) (DeSesso and Jacobson, 2001).

**Modifications in the small intestine lumen**

In humans, the small intestine lumen may contain sloughed off cells, intestinal secretions, partially digested food and a small number of micro-organisms (10⁴ to 10⁶ bacterial per ml, compared to the colon which contains 10¹² bacterial per gram). The small intestine contents may contain some hydrolytic enzymes capable of hydrolysing flavonoid glycosides, but this has not been demonstrated.

**Modifications in the small intestine epithelial cells**

A substantial amount is now known concerning the capacity of the small intestine epithelial cells to hydrolyse flavonoid glycosides and to conjugate the resulting aglycone. Glucosylated flavonoids and isoflavonoids are deglycosylated by lactase phlorizin hydrolase (Day et al., 2000), an enzyme which is located in the brush border of the small intestine and is responsible for lactose hydrolysis. The enzyme is outside the epithelial cells and so molecules can be deglycosylated in the lumen without first having to traverse the membrane (Mantei et al., 1988). The product is a free aglycone which can then diffuse into the epithelial cell either passively or by facilitated diffusion. Consequently, absorption of isoflavone glucosides in human subjects is not affected by pre-treatment with a microbial β-glucosidase, presumably because LPH in the small intestine catalyses the same reaction (Richelle et al., 2002). An alternative mechanism is that the glucoside is transported into the cell in an intact form by a sugar transporter such as SGLT1 (Othof et al., 2000; Gee et al., 2000) and is then deglycosylated by cytosolic β-glucosidase (Day et al., 1998). Both routes give rise to intracellular aglycone, and in fact intracellular free aglycone is found in tissues from rat small intestine after perfusion in vitro with either quercetin glucosides (Gee et al., 2000) or isoflavone glucosides (Andlauer et al., 2000b). However, the intracellular conjugation within the epithelial cells of the small intestine is clearly seen since these experiments also demonstrate that glucuronide (and to a lesser extent sulfate and methylated derivatives) are present in the vascular (serosal) side (Andlauer et al., 2000a; Gee et al., 2000), as well as in isolated tissue (Andlauer et al., 2000b). These results and others demonstrate that the small intestine epithelial cells are the major site of conjugation of flavonoids (Crespy et al., 1999; Kuhnle et al., 2000). The role of the small intestine in first pass metabolism has been reviewed for drugs (Lin et al., 1999). The conclusions are that there is only a limited drug metabolism capacity of the small intestine; for drugs the contribution to the overall metabolism is less important than the liver, unless a very small oral dose is given. This last statement is important since most dietary compounds when consumed as food are present in low amounts, which is consistent with the small intestine playing a major role in metabolism. For non-glcosylated flavonoids such as catechin, absorption into small intestine epithelial cells is likely to be due to passive or facilitated diffusion as demonstrated in the rat small intestine in situ (Donovan et al., 2001). The products in all above studies at the serosal (blood) side were flavonoid conjugates, irrespective of whether the test compound was quercetin, isoflavones or catechin.

**Escaping first pass metabolism**

Nevertheless, there are reports that some flavonoids escape first pass metabolism. Evidence for this is derived from the presence of unconjugated flavonoids in the plasma. However, this has only been shown for galloylated catechins and isoflavones under certain conditions, and is dose and time dependent. Table 1 shows the results of several studies where absence of conjugation has been examined (many studies add deconjugating enzymes so the information of overall conjugation is lost). In addition, food intake will have an effect, since bioavailability of drugs subject to significant first pass metabolism during absorption is increased after a meal (Lin et al., 1999) and this is demonstrated by studies on isoflavones in which plasma aglycones are only found in food-deprived, not fed, rats (Table 1). In addition, lipophilic compounds which pass into the lymph escape first pass metabolism, since lymph joins venous blood returning to the heart; however, it is unknown if flavonoids are transported in the lymph. Conjugated flavonoids would not be expected to enter the lymphatic system owing to their hydrophobicity, but it is conceivable that some aglycones might.