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Flavonoids: True or promiscuous inhibitors of enzyme? The case of deoxyxylulose phosphate reductoisomerase

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ABSTRACT

Flavonoids, due to their physical and chemical properties (among them hydrophobicity and metal chelation abilities), are potential inhibitors of the 1-deoxyxylulose 5-phosphate reductoisomerase and most of the tested flavonoids effectively inhibited its activity with encouraging IC_{50} values in the micromolar range. The addition of 0.01% Triton X100 in the assays led however, to a dramatic decrease of the inhibition revealing that a non-specific inhibition probably takes place. Our study highlights the possibility of erroneous conclusions regarding the inhibition of enzymes by flavonoids that are able to produce aggregates in micromolar range. Therefore, the addition of a detergent in the assays prevents possible false positive hits in high throughput screenings.

1. Introduction

Flavonoids are secondary plant metabolites essentially known for their antioxidant properties. They have also antiviral, antiprotozoal and interestingly antimicrobial activities [1–3], which are well reported in the PubChem BioAssays database [4]. Flavonoids inhibit many unrelated enzymes (see Table S1 for an extensive list of enzymes inhibited by these compounds). They can bind to enzymes according to different mechanisms owing to their multiple binding possibilities: hydrogen bonding, hydrophobic interactions, metal chelation and ππ-stacking, with a tendency to occupy hydrophobic pockets [5].

The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, involved in the biosynthesis of isoprenoids, constitutes a valuable target to conceive new antimicrobial drugs [6]. This pathway is notably present in some extremely virulent pathogens such as *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and *Plasmodium falciparum*, the parasite responsible for malaria [7,8]. The inhibition of this pathway is particularly attractive as it is absent in humans. The inhibition of 1-deoxyxylulose 5-phosphate reductoisomerase (DXR), the second enzyme of the pathway, is the most studied since the natural product fosmidomycin [9,10] was found to be an efficient inhibitor of this enzyme [11]. Unfortunately, bacteria become rapidly resistant, restricting its use in antibiotic therapies [12,13]. The syntheses of multiple fosmidomycin derivatives to improve its pharmacological properties gave rather disappointing results. Among the tested analogues, only few have similar or slightly better inhibition potency [14–19].

With the aim to find new hits, we tested the ability of flavonoids to inhibit the activity of the *E. coli* DXR (Fig. 1). Some considerations motivated this study. Firstly, their metal chelation potential lets envisage that they might chelate the Mg^{2+} cation present in the DXR active site. Flavonoids containing a catechol group were accordingly selected to optimize the number of chelation sites. Indeed, catechols form stable complexes with the magnesium dication Mg^{2+}, a hard metal ion [20]. In addition, flavonoids may act as potential efficient inhibitors as the DXR has hydrophobic pockets situated close to the active site where they might interact and interfere with the enzymatic activity [21]. Finally, considering their structures, a binding in the NADPH recognition site is also conceivable. Analysing a set of flavonoids with different structure should thus allow a structure–activity relationship study.

An aspect is however often neglected in enzyme inhibition studies with flavonoids, the possibility of the rather hydrophobic molecules to give aggregates in aqueous solution. Their formation depends not only on the flavonoid structures but also on their concentration and the nature of the buffer [22]. These aggregates are capable of inhibiting enzyme in a non-specific manner [23–26]. The inhibition seems to occur *via* a partial unfolding of the enzymes once they are bound to such aggregates [27]. Such

Abbreviations: MEP, 2-*C*-methyl-D-erythritol 4-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; H-DXR, His-tagged DXR; DXP, 1-deoxy-D-xylulose 5-phosphate.

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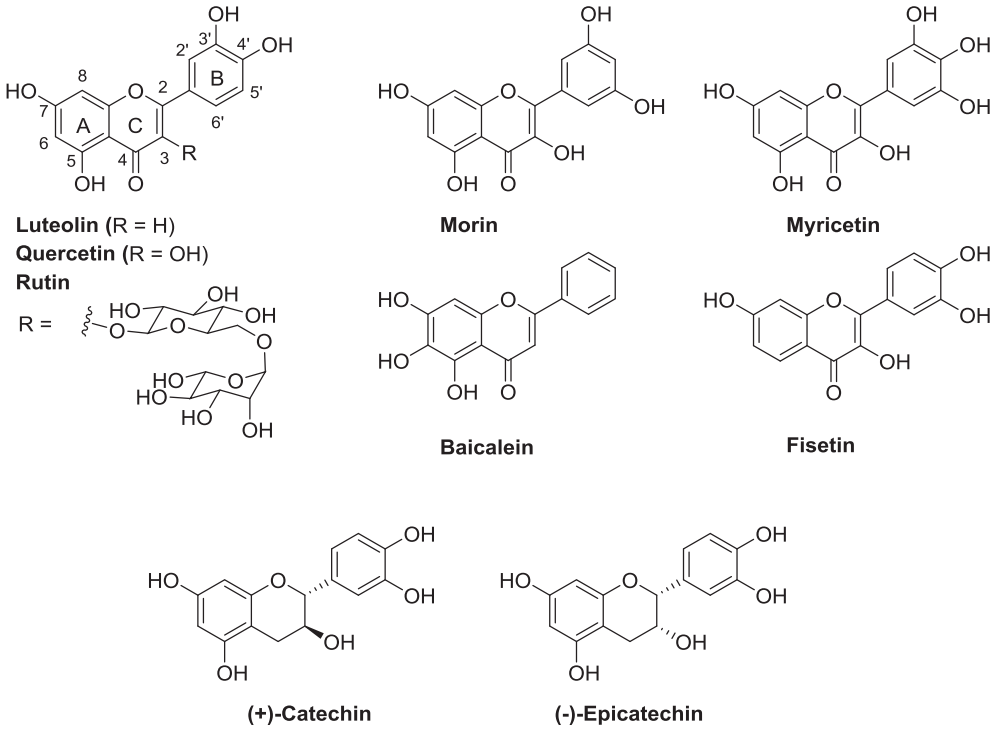

Fig. 1. Flavonoids tested on His-tagged DXR of *E. coli*.

inhibitors, also called aggregating or promiscuous inhibitors, are considered as "false positives" in a high throughput screening, and are unsuitable as lead compounds [28].

As some flavonoids have a promising potential to inhibit the bacterial DXR, the influence of selected flavonoids was analysed on the activity of His-tagged DXR of *Escherichia coli* (H-DXR). Discrimination between specific and non-specific inhibition by these flavonoids was attempted. A basic method is to compare the inhibition induced by the compounds in the presence and the absence of a non-ionic detergent [29,30]. A non-specific inhibition will be established when the detergent leads to a dramatic decrease of the inhibition level.

2. Results

Suspecting that flavonoids could be slow-binding inhibitors like fosmidomycin, we tested the influence of preincubation of quercetin on the DXR inhibition [31,32]. Clearly, a significant increase of the inhibition was observed when flavonoids were preincubated with DXR in the absence of NADPH. At a 20 μM concentration, the inhibition increased from 30% to 81% when the flavonoid was incubated during 2 min with DXP and H-DXR. Accordingly, the enzyme assays were all performed after a preincubation of the potential inhibitors with H-DXR. The inhibition of H-DXR by flavonoids is determined by the measure of IC_{50} or by calculating the percentage of inhibition (Table 1).

Among the tested flavonoids those possessing a catechol group on ring A or B inhibited the enzyme at a low micromolar concentration. Morin with a resorcinol group on ring B as well the flavan-3-ols (+)-catechin and (−)-epicatechin, while bearing a catechol group were much less efficient. Rutin, a 3-*O* glycosylated quercetin derivative did not inhibit H-DXR.

The presence of detergent led to a significant decrease of the inhibition potency (Table 1). It was obvious with baicalein where the inhibition was almost non-existent. When the concentration of flavonoids in the reaction medium varied, no clear dose-dependent inhibition was observed. For example with quercetin, the inhibition remained constant at a level of about 20–25% between 1 and 20 μM flavonoid concentrations. This observation might indicate that a specific inhibition, to some extent, by several flavonoids could not be excluded.

Table 1
Influence of the presence of 0.01% Triton X100 on the inhibition of H-DXR with flavonoids and related compounds.

	No Triton		With Triton
Flavonoids	IC_{50} (μM)	% Of inhibition	% Of inhibition
Luteolin	**3.4**	–	*37*[a]
Quercetin	**1.9**	–	*31*[a]
Rutin	–	*7*[b]	ND
Morin	–	*56*[b]	ND
Myricetin[#]	**1.5**	–	*27*[a]
Baicalein	**2.2**	–	*<5*[a]
Fisetin	**3.5**	–	*21*[a]
(+)-Catechin	–	*40*[b]	ND
(−)-Epicatechin	–	*52*[b]	ND
Catechol	–	*7*[b]	ND
Fosmidomycin[*]	**0.042**	–	–

Reactions were conducted as described in **Experimental**. When feasible, **IC_{50}** were determined (values in bold), otherwise the *percentages of inhibition* at an inhibitor concentration of [a] 20 μM or [b] 80 μM are given (values in italic) ND: not determined.
[*] The IC_{50} of fosmidomycin was determined as described in [31].
[#] Illustration of influence of Triton X100 on the inhibition of H-DXR with myricetin (Fig. S1).

As the compounds are susceptible to target the coenzyme binding site, we determined the influence of NADPH on the inhibition level (Table 2). Even at a saturating concentration of NADPH, the protection was only partial, suggesting that flavonoids probably do not bind to the NADPH binding site.

As the flavonoids did not seem to fit in the cofactor binding site, we studied the influence of the concentration of DXP on the inhibition of the enzyme by myricetin. Our kinetic studies revealed that myricetin behaves as a pure uncompetitive inhibitor of H-DXR *versus* DXP (Fig. 2). The replots (apparent $1/V_{max}$ and $1/K_m$ *versus* myricetin concentration, see Fig. S2) were linear allowing the