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Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids

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Abstract

The flavonoid family shows a high potential for inhibition of xanthine oxidase. Currently, more than 4000 flavonoids are known. The data of this study indicate that a planar structure is necessary for high inhibitory activity towards xanthine oxidase. Moreover, the contribution of a hydroxyl conjugate turns out to be a constant factor when the natural logarithm of IC_{50} values is taken. This finding allows us to accurately predict the IC_{50} value of any given hydroxyl group added to the basic flavone structure towards xanthine oxidase. This new method may provide an important research tool for elucidating the role that flavonoids may have in radical related diseases.

Keywords: Xanthine oxidase; Flavonoid; Structure–function relationship; Inhibition; IC_{50}

1. Introduction

Use of plants for health improvement goes back to Chinese medicine 4000–6000 years ago, long before active compounds such as flavonoids were identified (Gabor, 1986; Selway, 1986).

Numerous positive effects of flavonoids have been described, such as antioxidant (Middleton, 1998; Nijveldt et al., 2001; Rice-Evans, 1995), antiviral (Middleton, 1998; Selway, 1986), anticancer (Ames et al., 1995; Fujiki et al., 1986; Middleton, 1998; Plaumann et al., 1996), anti-inflammation (Hayek et al., 1997), antiallergic (Gabor, 1986; Middleton, 1998), anti-atherogenesis (Hayek et al., 1997), antithrombosis and others (Baeuerle et al., 1996; Berger et al., 1992).

Next to their antioxidant capacity, flavonoids can contribute to a decrease in oxidative stress via inhibition or activation of key regulating enzymes (Adkins and Taylor, 1990; Koyama et al., 1999; Matsumura et al., 1998; Nielsen et al., 1996) such as xanthine oxidase, phospholipase and nitric oxide synthase. Furthermore, flavonoids may inhibit peroxynitrite formation by inhibiting inducible nitric oxide in activated macrophages, which is enhanced under certain stress conditions (Kim et al., 1999; Liang et al., 1999).

One very important enzyme that has been reported to increase during oxidative stress is xanthine oxidase. Therapeutic use of inhibitors of xanthine oxidase has been proposed in the prevention of ischemia–reperfusion injury (Adkins and Taylor, 1990; Hearse et al., 1986; Rose et al., 1998). There are five different mechanisms known to increase superoxide generation by xanthine oxidase during ischemia–reperfusion.

Granger et al. (1981) first suggested that during ischemia–reperfusion, superoxide and hydrogen peroxide production is enhanced due to increased conversion of xanthine dehydrogenase to xanthine oxidase . This hypothesis has been confirmed (Rasmussen et al., 2000; Saksela et al., 1999). Secondly, mRNA levels of xanthine dehydrogenase and xanthine oxidase are upregulated (Saksela et al., 1998). Thirdly, when the liver becomes ischemic and hepatocellular damage occurs, the liver releases xanthine oxidase in the

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bloodstream (Sanhueza et al., 1992), this superoxide-producing enzyme thus being transported throughout the body. Further, xanthine oxidase can specifically bind to endothelial cells and cell-bound xanthine oxidase has been reported to produce radicals, which are inaccessible to CuZn–superoxide dismutase (Houston et al., 1999).

Finally, during ischemia, ATP is degenerated to xanthine and hypoxanthine, thereby increasing xanthine oxidase substrate levels, which leads to increased superoxide production.

If these radicals exceed the defense mechanism of the body (e.g. superoxide dismutase, catalase, etc.), then superoxide may yield highly reactive products such as H_2O_2 and, in the presence of Fe^{2+}, to HO_2°, hydroyl and peroxyl radicals. These reactive species can disturb normal cell physiology, eventually leading to cell death and multiple organ failure (Anup and Balasubramanian, 2000; Papathanassoglou et al., 2000).

The enormous amount of data published suggests that flavonoids may provide a remedy in radical-mediated conditions at various stages of oxidative stress (Halliwell, 1995; Kim et al., 1999; Manach et al., 1999; Matsumura et al., 1998; Sanhueza et al., 1992). Xanthine oxidase is recognized as an important enzyme in radical-mediated diseases. However, because the flavonoid family represents more than 4000 different compounds, there is a need for clarification of structure–function relationships in order to find the most active compound. However, there are so far only few studies that explored the structure–function relationships of flavonoids to classify their potential capacity towards xanthine oxidase inhibition. Although the fine studies carried out by Cos et al. and Nagao et al. are appropriate to show the importance of the hydroxyl group location on the flavone aglycone, they lack specificity regarding the actual importance of a single hydroxyl group to the xanthine oxidase IC_{50} value. Such an approach still makes it necessary to measure a huge amount of the total flavonoid pool. Recently, a computer program-based attempt was made to classify the capacity of different flavonoids for xanthine oxidase inhibition in order to save time. Unfortunately, this approach was only able to classify the flavonoids from weak to strong inhibitor without any data on their possible IC_{50} value towards xanthine oxidase inhibition. This approach was thus unusable for the screening of potentially active

Table 1
Xanthine oxidase inhibition by various flavonoid subclasses

Subclass	Systemic name	OH substituent	Compound number	IC_{50} value[a]	Prediction of IC_{50}[a]	Accuracy (%)
Flavone		–	**1**	>40	1638	–
		3	**2**	>40	33832	–
		4′	**3**	>40	108	–
		5	**4**	>40	20	–
		6	**5**	>40	nc	–
		7	**6**	40	39.0	97.5
	Chrysin	5,7	**7**	2.5	2.6	96.2
		6,3′	**8**	>40	nc	–
		5,2′	**9**	>40	1934	–
		7,8	**10**	>40	173	–
		3′,4′	**11**	40	48	83.3
		6,2′	**12**	>40	nc	–
	Galagin	3,5,7	**13**	4.0	7.3	54.8
		7,3′,4′	**14**	4.0	4.5	88.9
	Apigenin	5,7,4′	**15**	1.0	1.1	90.9
	Luteolin	5,7,3′,4′	**16**	0.75	0.75	100.0
	Fisetin	3,7,3′,4′	**17**	11.3	11.0	97.3
		7,8,3′,4′	**18**	10.0	10.6	94.3
	Kaempferol	3,5,7,4′	**19**	2.5	2.2	88.0
	Quercetin	3,5,7,3′,4′	**20**	1.5	1.4	93.3
	Myricetin	3,5,7,3′,4′,5′	**21**	1.5	1.4	93.3
	Morin	3,5,7,2′,4′	**22**	40	22.3	55.8
Flavanone	Taxifolin	3,5,7,3′,4′	**23**	>40	nc	–
Isoflavone	Daidzein	7,4′	**24**	>40	nc	–
	Genistein	5,7,4′	**25**	>40	nc	–
Catechins	Epicatechin	3,5,7,3′,4′	**26**	>40	nc	–
	Epigallocatechingallate	5,7,3′,4′,5′;3-*O*-galloyl	**27**	>40	nc	–
	Allopurinol[b]		**28**	6.2	nc	–

nc = no calculation possible for this compound, because none of the compounds tested with this specific hydroxyl group has an IC_{50} below or equal to 40 μM. Indicated are IC_{50} values of flavones towards xanthine oxidase, both measured and predicted. The predictive method is based upon contribution of additive hydroxyl moieties to a certain flavone structure by taking the average natural logarithm of the IC_{50} value. This predictive method is furthermore based upon the average natural logarithm of contribution factors of the different hydroxyl moieties.

[a] IC_{50} value is expressed in μM.

[b] Allopurinol is a pharmaceutical component, not a member of the flavonoid family, commonly used as a xanthine oxidase inhibitor.

flavonoids. However, our data clearly indicate that bioactive properties can be restricted to a subclass of the flavonoid family and that even in this subclass, there are large differences, which are structure dependent. A thorough structure–function relation study will be of help in selecting the flavonoids most active in radical-mediated conditions, but a method for predicting activity is even more attractive, since this will avoid the need for laborious experimental work. Based on structure–function relationships presented, we describe a method for predicting IC_{50} values of xanthine oxidase very accurately by calculating the contribution of each hydroxyl moiety function towards inhibition of this enzyme. This very attractive predictive method can be used to select the most active inhibitors out of the large pool of flavonoids.

2. Materials and methods

All flavonoids used were obtained from Indofine chemicals, except for Epicatechin and Epigallocatechin gallate, which were obtained from Sigma.

2.1. Xanthine oxidase activity measurement

The activity of xanthine oxidase is measured by uric acid formation monitored at 295 nm. The reaction mixture contains 724 μl of 50 mM K_2HPO_4 pH 7.8 and 200 μl of 84.8 μg/ml xanthine in 50 mM K_2HPO_4. The reaction is started by addition of 66 μl 37.7 mU/ml xanthine oxidase. The reaction is monitored for 6 min at 295 nm and the product is expressed as μmol uric acid per minute. The reaction kinetics were linear during these 6 min of monitoring. The inhibition of xanthine oxidase by flavonoids is expressed as the concentration that results in half-maximal enzyme velocity (IC_{50}).

2.2. Calculation of the contribution of hydroxyl moieties to xanthine oxidase inhibition

The contribution of a hydroxyl group can be calculated as follows:

$$\ln(IC_{50\ \text{flavone without this moiety}} \times 10) \Big/ \ln(IC_{50\ \text{flavone with this moiety}} \times 10).$$

IC_{50} values were multiplied by a factor of 10 to avoid negative values. As ratios are used and not exact values, this multiplication does not influence the prediction of IC_{50} values (Fig. 3).

2.3. Calculation of predictive IC_{50} values of flavones

Predictive values were obtained by first calculating the inhibitory activity of the flavone backbone (Table 1). Then, the IC_{50} value of each flavone was calculated on the basis of the median contribution of each hydroxyl function (Table 2). The IC_{50} value of the flavone backbone is the median of the values calculated for quercetin, apigenin and luteolin.[1]

Table 2
Contribution of different hydroxyl moieties to IC_{50} values indicated as ln IC_{50} ratios

OH substituent	ln ratio	Compounds compared
3	0.87	**7** vs. **13**
	0.78	**14** vs. **17**
	0.72	**15** vs. **19**
	0.74	**16** vs. **20**
5	1.86	**6** vs. **7**
	1.83	**14** vs. **16**
	1.75	**17** vs. **20**
7	1.62	**11** vs. **14**
8	0.80	**14** vs. **18**
2′	0.54	**19** vs. **22**
3′	1.14	**15** vs. **16**
	1.19	**19** vs. **20**
4′	1.40	**7** vs. **15**
	1.15	**13** vs. **19**
5′	1.00	**20** vs. **21**
3,5	1.62	**6** vs. **13**
	1.36	**14** vs. **20**
3,7	1.27	**11** vs. **17**
3,2′	0.38	**15** vs. **22**
3,3′	0.85	**14** vs. **20**
3,4′	1.00	**7** vs. **19**
5,7	2.97	**11** vs. **16**
5,4′	2.60	**6** vs. **15**
7,8	1.30	**11** vs. **18**
2′,4′	0.62	**13** vs. **22**
3′4′	1.36	**13** vs. **20**
	1.60	**7** vs. **16**
	1.62	**6** vs. **14**

ln ratio is calculated as follows: ln ((IC_{50} compound 1) × 10)/ln ((IC_{50} compound 2) × 10).

3. Results

Xanthine oxidase, which has been reported to increase its activity during oxidative stress (Adkins and Taylor, 1990; Koyama et al., 1999; Manach et al., 1999; Matsumura et al., 1998; Sanhueza et al., 1992), produces uric acid and superoxide (Fig. 1). Inhibition of this enzyme is measured by decreased uric acid production. Flavonoids from four different subclasses (Fig. 2) were tested. From these four, only the flavones expressed xanthine oxidase inhibitory capacity. Consequently, this subclass was explored further (Table 1).

The strongest contribution towards xanthine oxidase inhibition results from introduction of a 5-hydroxyl or

[1] For example, the calculation of the IC_{50} of the flavone backbone based on luteolin is: exp(ln IC_{50} luteolin (0.75 × 10)) × (median of 5OH(1.83) × 7OH(1.62) × median 3′,4′ (1.60))/10 is 1382 μM.

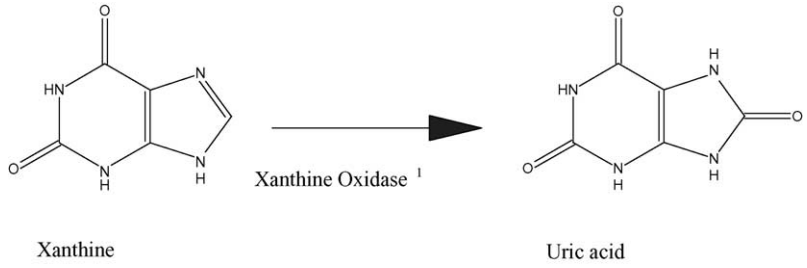


Fig. 1. Xanthine oxidase assay. [1]Assay used in the present study, uric acid formation measured spectrophotometricaly.

7-hydroxyl moiety to a flavone backbone. For example, introduction of a 5-hydroxyl moiety decreases IC_{50} by a factor of 16 (**6** vs. **7**), 7.5 (**17** vs. **20**) and 5.3 (**14** vs. **16**). Introduction of a 7-hydroxyl group results in a decrease by a factor of 10 (**11** vs. **14**). Moreover, 7-hydroxyflavone is the only flavone with one hydroxyl group that has measurable (≤ 40 μM) xanthine oxidase inhibitory capacity, whereas the other flavones with one hydroxyl moiety have IC_{50} values substantially higher than 40 μM. A smaller but significant decrease in IC_{50} value is seen when a 3′- or 4′-hydroxyl group is added. For the 3′-hydroxyl moiety the IC_{50} value decreases from 1 to 0.75 μM when apigenin is compared with luteolin (**15** vs. **16**) and it changes from 2.5 to 1.5 μM when kaempferol is compared with quercetin (**19** vs. **20**). Introduction of a 4-hydroxyl subunit results in an increase of inhibition by a factor of 2.5 (**7** vs. **15**) or 1.5 (**13** vs. **19**). To illustrate the contribution of the 5,7,3′ and 4′ hydroxyl functions to xanthine oxidase inhibition luteolin (**16**) which has all the abovementioned hydroxyl groups has the lowest IC_{50} value.

Some hydroxyl groups show negative effects on IC_{50} values. The group with the strongest negative contribution to inhibition of xanthine oxidase is the 2′-hydroxyl moiety as can be concluded from comparing kaempferol (**19**) with morin (**22**) in which the IC_{50} increases from 2.5 to 40 μM, respectively. Presence of a 3-hydroxyl moiety also attenuates inhibition as can be concluded from comparing luteolin (**16**) with quercetin (**20**) showing an increase of IC_{50} from

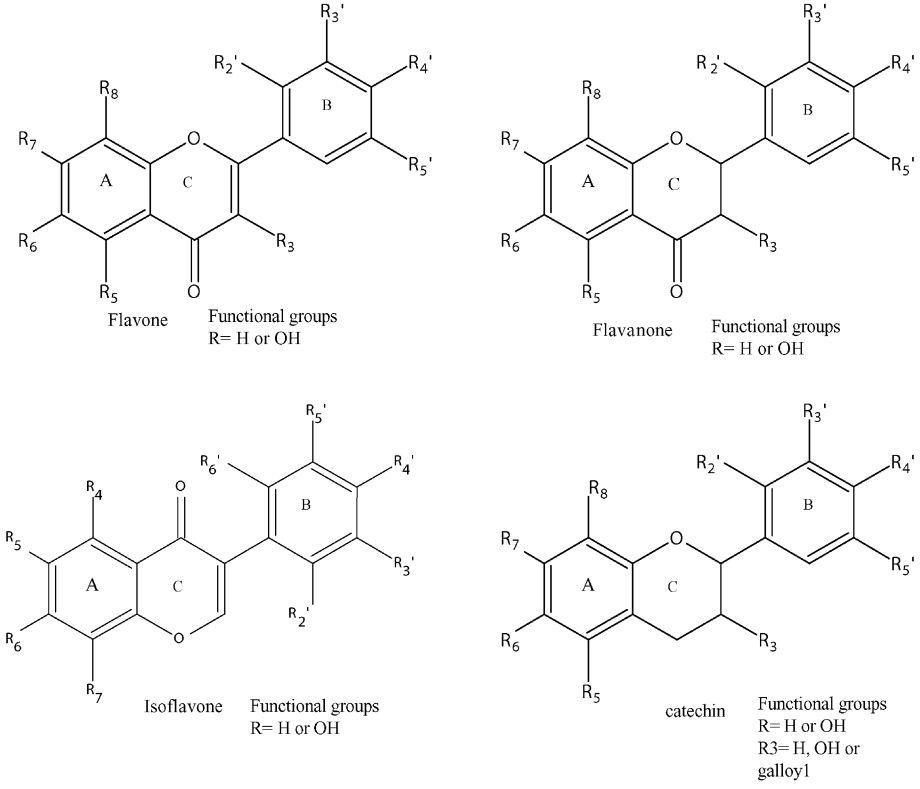


Fig. 2. Structures of different flavonoid subclasses.

Table 3
Prediction for two flavones not included in the setup of the calculation method

Subclass	Systemic name	OH substituent	Compound number	IC_{50} value[a]	Prediction of IC_{50}[a]	Accuracy (%)
Flavone	Resokaempferol	3,7,4′	**29**	22	25.0	88.0
	Robinetin	3,7,3′,4′,5′	**30**	9	11.4	79.9

The predictive method has an average accuracy of 83% for these flavonoids.
[a] IC_{50} value is expressed as μM.

0.75 to 1.5 μM, respectively. Also, an 8-hydroxyl substituent was found to have a negative effect on xanthine oxidase inhibition. Addition of an 8-hydroxyl moiety increases IC_{50} from 4 to 10 μM when 7,3′,4′-trihydroxyflavone (**14**) is compared to 7,8,3′,4′-tetrahydroxyflavone (**18**). Addition of a 5′-hydroxyl moiety has no effect on xanthine oxidase inhibition.

As can be seen from the examples mentioned above, the contribution factor of an extra hydroxyl substituent is not constant, but seems to depend on the IC_{50} value of the flavone involved. Surprisingly, when the natural logarithm of IC_{50} values of flavones is calculated, the contribution of a hydroxyl moiety toward xanthine oxidase inhibition becomes a constant factor (Table 2). When, for instance, a 5-hydroxyl group is introduced, this results in a ln ratio of 1.86 (**6** vs. **7**), 1.83 (**14** vs. **16**) and 1.75 (**17** vs. **20**), while the ratios based on IC_{50} value mentioned in Table 1 are 16, 5.3 and 7.5, respectively.

Based on these calculations, a ranking of hydroxyl groups contributing to IC_{50} values can be made. Introduction of a 5-hydroxyl moiety results in the strongest contribution towards xanthine oxidase inhibition. Similar effects are found when a 7-hydroxylfunction is introduced. To a minor extent, the 3′- and 4′-hydroxyl moieties result in a decrease of IC_{50} values. The effect of addition of both a 3′- and a 4′-hydroxyl moiety has approximately the same effect as addition of either a 5- or 7-hydroxyl group alone.

These constant contributions of hydroxyl moieties to inhibition of xanthine oxidase open possibilities for predicting inhibition constants. To investigate this interesting option, we first calculated the IC_{50} value of the flavone backbone by subtracting the contribution of the hydroxyl functions of luteolin (**16**), apigenin (**15**) and quercetin (**20**) resulting in a backbone IC_{50} value of 1638 μM^2. Based on this flavone skeleton, IC_{50} values of other flavones were calculated (Table 1), showing clearly that the calculation of inhibition constants is very accurate. To test our method further, we calculated IC_{50} values for two flavones not used for building our model calculation. As can be concluded from Table 3, prediction for these flavones was also very accurate (83%). The data presented here indicate for the first time that IC_{50} values for xanthine oxidase inhibition by flavones can be predicted.

This prediction of inhibition constants based on ln ratios drew our attention to the mechanism. The Lineweaver–Burk plot for quercetin (**20**) (Fig. 3) showed that inhibition of xanthine oxidase is competitive, which was also seen for other flavones (data not shown), suggesting binding of flavones to xanthine oxidase allosteric center, thereby replacing xanthine. Allopurinol also showed competitive inhibition. Morin (Fig. 4) and galagin both showed mixed type inhibition, which could possibly explain the large difference between measured IC_{50} value and predicted IC_{50} value.

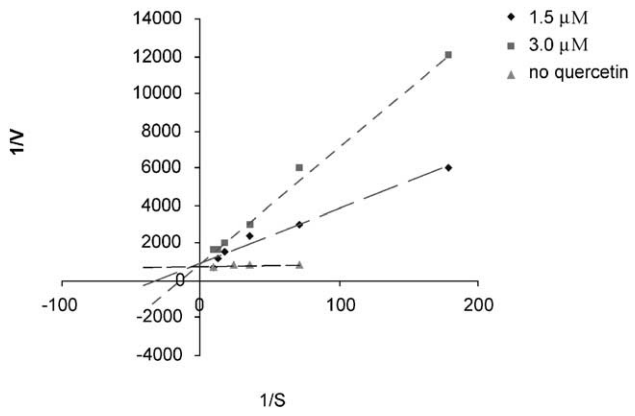


Fig. 3. Lineweaver–Burk plot for quercetin. The inhibition of xanthine oxidase by quercetin is competitive, as can be seen from similar V_{max} and distinctly different K_m values.

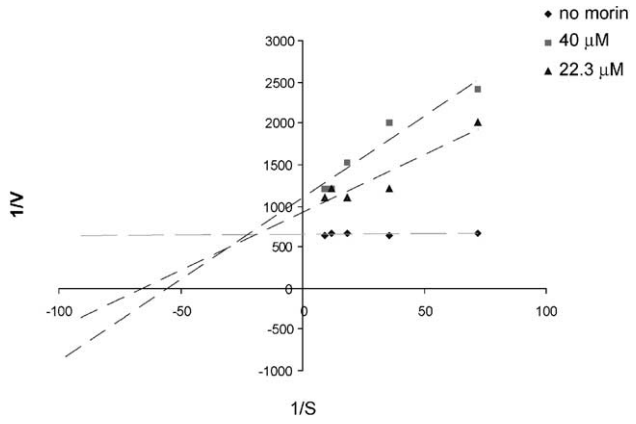


Fig. 4. Lineweaver–Burk plot for morin. Inhibition of xanthine oxidase by morin shows mixed type inhibition, as can be concluded from different V_{max} and different K_m values.

4. Discussion

Increased xanthine oxidase activity is assumed to be important in mediating oxidative injury in ischemia–reperfusion (Adkins and Taylor, 1990; Manach et al., 1999; Matsumura et al., 1998; Sanhueza et al., 1992). Allopurinol is an allosteric xanthine oxidase inhibitor, which can decrease the damaging effect of xanthine oxidase in radical-mediated diseases. Use of allopurinol is restricted by formation of oxypurinol, which is known to cause side effects (Hamanaka et al., 1998). Flavonoids might provide an interesting alternative for treatment of radical-mediated diseases (Adkins and Taylor, 1990; Hayek et al., 1997; Koyama et al., 1999; Manach et al., 1999; Matsumura et al., 1998; Plaumann et al., 1996; Sanhueza et al., 1992). The enormous diversity of the flavonoid family suggests the need for a knowledge of structure–function relationship to select the most active candidates.

Calculation of ln IC_{50} ratios reveals that there are four hydroxyl groups positively contributing to inhibition. The relative importance of induction of extra hydroxyl substituents is in decreasing order 5>7>4′=3′. As can be concluded from these calculations, luteolin (**16**), having all four beneficial hydroxyl moieties, should have the highest inhibitory activity, as shown clearly in Table 1. Unexpectedly, 7-hydroxyflavone (**6**) is a better inhibitor than 5-hydroxyflavone (**4**), which is in contradiction to results mentioned above that show a higher contribution factor for a 5-hydroxyl moiety. A possible explanation for this difference will be discussed in the section about the xanthine oxidase allosteric center.

Hydroxyl groups, which make a negative contribution to the inhibition of xanthine oxidase, are in the 2′, 8 and 3 position. Substitution of a hydroxyl group at the 2′ position has the greatest impact. The effect of introduction of a hydroxyl function at position 8 and 3 is approximately the same. Negative effects on inhibition might be due to disruption of hydrogen bonds within the allosteric center due to steric hindrance.

Although Cos et al. demonstrated the importance of a 5- and 7-hydroxyl moiety, a closer look at their data revealed the importance of the 3′- and 4′-hydroxyl moiety, which is consistent with our report. When chrysin (**7**) is compared with luteolin (**16**), a small but distinct difference is seen, demonstrating the influence of a 3′- and 4′-hydroxyl moiety on xanthine oxidase inhibition.

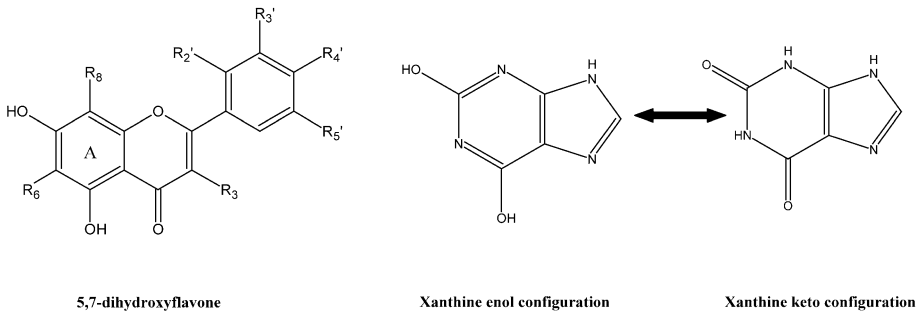
The relative contribution of hydroxyl conjugates to xanthine oxidase inhibition could be calculated by using the natural logarithm (ln) of IC_{50} values (Table 2). Surprisingly, the ln IC_{50} ratios were found to be constant and can be used to predict IC_{50} values of all members with a flavone backbone. The predictive value of the calculation method was further supported by the accurate prediction of IC_{50} values of other flavones (Table 3) not used to build our model. To our knowledge, this is the first published method for successful prediction of IC_{50} values of flavones.

When our method is applied to results published by Nagao et al., calculation of contribution factors of various hydroxyl moieties clearly shows the versatility of our method. Based on published data, contribution factors of 1.3 and 1.7 were calculated for the 3′-hydroxyl function and 3′,4′-dihydroxyl group, respectively, both of which are in line with our data (1.2 and 1.6, respectively). Using our method for calculation based on data published by Cos et al. (1998) gave disappointing results. Calculation of the 5-hydroxylgroup, for instance, resulted in a contribution factor of ranging from 2, 79–1, 15. This inconsistency of the contribution factor was probably caused by use of different assays.

Recently, Ponce et al. (2000) presented a computer program for clarifying as to their ability to inhibit xanthine oxidase. Unfortunately, using this method, only qualitative prediction of inhibition was possible, which is in contradiction to our method which gives excellent quantitative results.

This calculation and prediction of IC_{50} values drew our attention to mechanistic aspects of xanthine oxidase inhibition by flavones. The Lineweaver–Burk plot for quercetin shows clearly competitive inhibition kinetics (Fig. 3), indicating that flavones bind in the same redox center as xanthine, which is suggested to be the molybdenum center (Kim and Hille, 1993; Lorigan et al., 1994).

In the present study, we demonstrated clearly that a planar flavonoid structure is necessary for optimal xanthine



5,7-dihydroxyflavone Xanthine enol configuration Xanthine keto configuration

Fig. 5. Comparing structure similarities of xanthine enol form with 5,7-dihydroxylflavone. Structural similarity between xanthine enol form and 5,7-dihydroxylflavone suggests a similar binding location in xanthine oxidase allosteric center.

oxidase inhibition. This finding is in accordance with results of previous studies carried out by Cos et al. (1998) and Nagao et al. (1999). Necessity for a planar structure is clearly illustrated by comparing taxifolin (**23**) with quercetin (**20**), which shows that introduction of a double bond in the C ring, forming a planar structure, results in increased inhibition (>40 μM vs. 1.5 μM, respectively).

Enroth et al. (2000) recently published the crystal structures of bovine milk xanthine oxidase. They suggested that phe 1009 interacts with the six-membered ring of xanthine and phe 914 interacts with the five-membered ring of xanthine. It may be hypothesized that the planar A and C ring of flavones have π–π interactions with phe 1009 and phe 914, which are necessary for inhibition of xanthine oxidase. A planar flavone skeleton alone is insufficient to induce xanthine oxidase inhibition. It may be speculated that at least one hydroxyl group is necessary, favorable at position 7, to achieve xanthine oxidase inhibition by flavones. Addition of a 5-hydroxyl moiety in the presence of a 7-hydroxyl moiety decreases IC_{50} value dramatically (**6** vs. **7**), this effect may be explained by structural similarities between 5,7-dihydroxyflavone (A ring) and the six-membered ring of xanthine in enol form (Fig. 5). The other positively contributing hydroxyl groups may increase affinity by additional hydrogen bond formation.

In conclusion, we now described a new and successfully applied concept to accurately predict IC_{50} values of flavones based upon an individual contribution factor dependent on the location of a hydroxyl moiety in the flavone skeleton. This predictive method may provide an important tool for research into flavonoids for application in radical-related diseases. We assume that predicting an IC_{50} value on the basis of structural features will prove to be eminently valuable for selecting the best enzyme inhibitors for many different enzymes with a minimum of measurements and expeuse.

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