

St. John's Wort Extracts and Some of Their Constituents Potently Inhibit Ultimate Carcinogen Formation from Benzo[*a*]pyrene-7,8-Dihydrodiol by Human CYP1A1

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ABSTRACT

Commercially available St. John's wort (*Hypericum perforatum*) preparations and some of their main constituents (hypericin, pseudohypericin, hyperforin, rutin, and quercetin) were examined for their potential to inhibit carcinogen activation by human cytochrome P450 1A1 (CYP1A1). We used a reconstituted system consisting of purified human CYP1A1, purified human NADPH-cytochrome P450 reductase, and dilaurylphosphatidylcholine as lipid component. St. John's wort extracts potently inhibited CYP1A1-catalyzed (\pm)-*trans*-7,8-dihydro-7,8-dihydroxy-benzo(*a*)pyrene (7,8-diol-B[*a*]P) epoxidation, the terminal reaction leading to the ultimate carcinogenic product (\pm)-B[*a*]P-r-7,t-8-dihydrodiol-t-9,10-epoxide (diolepoxide 2). All constituents, except rutin, were shown to possess strong inhibitory potencies toward diolepoxide 2 formation from 7,8-diol-B[*a*]P, with IC₅₀ values of 0.5 μ M (hypericin), 1.2 μ M (hyperforin), 1.5 μ M (quercetin), and 8 μ M (pseudohypericin), respectively. Preincubation experiments revealed that their action was not mechanism based. Inhibition kinetics studies showed the anthrodianthrone compound hypericin to be a noncompetitive inhibitor, with a K_i value of 0.6 μ M, and the phloroglucinol hyperforin to be a competitive inhibitor, with a K_i value of 1.1 μ M. When the effects on NADPH-P450 reductase activity were investigated, all constituents of St. John's wort studied turned out to be rather ineffective inhibitors; quercetin was the only exception, with an IC₅₀ value of \sim 20 μ M. These *in vitro* data indicate that St. John's wort extracts and some of their constituents potently inhibit the major human procarcinogen-activating enzyme CYP1A1.

INTRODUCTION

St. John's wort (*Hypericum perforatum*) preparations are increasingly popular in the treatment of depression (1). They contain a complex mixture of approximately seven groups of biologically active phytochemicals, mainly polyphenols such as flavonol glycerides, flavonols and biflavones, naphthodianthrone, phloroglucinols, oligomeric proanthocyanides and tannins, phenylpropanes, and xanthenes (2, 3). Recent studies tried to identify the bioactive constituents that are responsible for therapeutic effects and drug-drug interactions. Many studies have demonstrated antiviral and antiproliferative activity of hypericin and its potentiation by light exposure (4–6). Hyperforin has been presumed as an active component in the treatment of depression by inhibiting serotonin, γ -aminobutyric acid, and L-glutamate uptake (7). Furthermore, it was found that hyperforin induced CYP3A4 by binding to the pregnane X receptor (8), whereas the expression of other CYP3A isoforms remained unaffected (9). It has been demonstrated that hypericum extracts and some of their constituents inhibit several important human P450¹ enzymes involved in the

metabolism of drugs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4; Refs. 10–12) and induce the multiple-drug resistance gene (*MDR 1*) product P-glycoprotein (13). Recent studies summarize our knowledge of reported and possible St. John's wort drug interactions (14, 15).

Little is known about the effects of St. John's wort on P450-mediated (xenobiotic) metabolism of dietary toxicants and environmental pollutants such as procarcinogens. Human CYP1A1 is one of the major P450 enzymes involved in chemical carcinogenesis; it is induced by polycyclic aromatic hydrocarbons such as B[*a*]P and activates them and many other environmental toxicants and procarcinogens to their ultimate carcinogenic form. In the case of B[*a*]P, the highly reactive diolepoxides 2, which can bind to DNA to initiate tumorigenesis, are formed (16–21). Thus, the inhibition of CYP1A1 may be one of the key mechanisms for cancer prevention.

In vitro characterization and assessment of the inhibitory capacity of natural polyphenols on CYP1A1 activity have been performed almost exclusively by examination of the *O*-dealkylation of alkoxyresorufins or similar model substrates. More recent studies reported potent and selective inhibition of human CYP1A subfamily P450s by the flavonoid galangin, the alkaloid rutaecarpine, and the stilbenes resveratrol, rhapontigenin, and tetramethoxy-stilbene (22–27). We found evidence, however, that the use of the EROD assay alone may lead to an incomplete assessment of the inhibitory potency of a substance that could be better evaluated in certain cases with the help of the diolepoxidation of 7,8-diol-B[*a*]P, the terminal step in the bioactivation of B[*a*]P leading to the ultimate carcinogenic product, diolepoxide 2 (28).

In the present study, we characterize, for the first time, the inhibitory potency of several St. John's wort preparations and their major constituents by their effects on the CYP1A1-mediated epoxidation of 7,8-diol-B[*a*]P. Activities were determined in an *in vitro* reconstituted system consisting of purified human CYP1A1, human P450 reductase, and dilaurylphosphatidylcholine as lipid. Results were compared with those of the EROD assay and the AHH test.

MATERIALS AND METHODS

Materials. Three commercially available St. John's wort preparations were used in this study: Jarsin-coated tablets (Lichtwer-Pharma, Berlin, Germany), Aristo drops (Steiner & Co., Berlin, Germany), and Esbericum capsules (Schaper & Brümmer, Salzgitter, Germany). They were dissolved in DMSO and tested directly for inhibition of CYP1A1, as described below. Quercetin, rutin, α -naphthoflavone, B[*a*]P, 7-ethoxyresorufin, resorufin, and dilaurylphosphatidylcholine were purchased from Sigma (Deisenhofen, Germany). Hypericin, hyperforin, and pseudohypericin were from Calbiochem (Bad Soden, Germany). 7,8-Diol-B[*a*]P and tetraols 1–4 were purchased from NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, MO). The tetraols were kindly provided by Prof. K.-L. Platt, and 3-OH-B[*a*]P was a gift from Dr. A. Seidel (both from the Institute of Toxicology, University of Mainz, Mainz, Germany).

7-ethoxyresorufin *O*-deethylation; AHH, aryl hydrocarbon hydroxylation; DE1, diolepoxide 1, (\pm)-B[*a*]P-r-7,t-8-dihydrodiol-c-9,10-epoxide; DE2, diolepoxide 2, (\pm)-B[*a*]P-r-7,t-8-dihydrodiol-t-9,10-epoxide; tetraol 1, r-7,t-8,t-9,c-10-tetrahydroxy-7,8,9,10-tetrahydro-B[*a*]P; tetraol 2, r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydro-B[*a*]P; tetraol 3, r-7,t-8,c-9,t-10-tetrahydroxy-7,8,9,10-tetrahydro-B[*a*]P; tetraol 4, r-7,t-8,c-9,c-10-tetrahydroxy-7,8,9,10-tetrahydro-B[*a*]P; HPLC, high-performance liquid chromatography.

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¹ The abbreviations used are: P450, cytochrome P450; CYP1A1, human cytochrome P450 1A1; P450 reductase, human NADPH-cytochrome P450 reductase; B[*a*]P, benzo[*a*]pyrene; 7,8-diol-B[*a*]P, (\pm)-*trans*-7,8-dihydro-7,8-dihydroxy-B[*a*]P; EROD,

Cloning, Heterologous Expression, and Purification of Recombinant Enzymes. Recombinant heterologously expressed human CYP1A1 and P450 reductase were generated in-house using a baculovirus/insect cell expression system. Human CYP1A1 was heterologously expressed as COOH-terminal His-fusion protein in *Spodoptera frugiperda* insect cells (Sf9) and purified as described (29). Purification with Ni-chelate chromatography resulted in an electrophoretically homogeneous CYP1A1 with a specific P450 content of 11 nmol of P450/milligram of protein that was almost free of cytochrome P420. Human P450 reductase was also heterologously expressed in insect cells and purified as described (30); the specific catalytic activity was 18.2 units/mg protein, and it appeared electrophoretically homogeneous according to SDS-PAGE.

Epoxidation Assay. 7,8-Diol-B[a]P epoxidation was determined based on methods described previously (31, 32, 28), with the exception that we used a reconstituted CYP1A1 system. A mixture of 200 pmol of purified CYP1A1 (28 nmol/ml), 900 pmol of purified P450 reductase (56 nmol/ml), and 0.5 mg of dilaurylphosphatidylcholine (5 mg/ml) was incubated in 200 μ l of assay buffer [50 mM Tris and 100 mM NaCl (pH 7.5)] on ice for 10 min. Then 10- μ l aliquots corresponding to 10 pmol of CYP1A1 were mixed with the substrate 7,8-diol-B[a]P (final concentration, 2 μ M) and, with the inhibitor dissolved in 200 μ l of buffer, diluted with buffer to a final volume of 990 μ l and preincubated at 37°C for 1 min. The reactions were started by the addition of 10 μ l of NADPH (16.6 mg/200 μ l) and were performed at 37°C in a shaking water bath for 15 min. The reactions were terminated by adding 100 μ l of cold 1 M potassium phosphate buffer (pH 3.5), and samples were placed at 4°C for 1 h. After that, 10 μ l of methanol containing 10 μ g of 1,1'-bi-2-naphthol as an internal standard and 2.5 ml of ethylacetate were added; the mixture was vortexed for 1 min and centrifuged for 3 min. The extraction procedure was performed twice. The top phases were combined and evaporated, and the residuals were redissolved in 100 μ l of methanol, sonicated in an ultrasonication bath, and injected into the HPLC apparatus.

The final DMSO content was <0.5% during incubation, and inhibition was assessed by comparison with controls containing the corresponding amount of DMSO. All assays were performed under dim light to avoid the well-known photosensitization reactions by hypericin.

HPLC Analysis. 7,8-Diol-B[a]P metabolism was analyzed by using a HPLC system (Shimadzu, Duisburg, Germany) consisting of two solvent delivery systems (LC-10 AD VP, a UV detector SPD-10 A VP) and run with CLASS VP software. Separation was performed with a reversed-phase column (Nucleosil 100-5 C18, 5 μ M, 250 \times 4 mm; Macherey-Nagel, Düren, Germany) at 40°C with a flow rate of 0.7 ml/min using a linear methanol-water gradient of 40% methanol rising to 47.2% in 35 min, followed by isocratic elution with 60% methanol for another 40 min and by 100% methanol for 10 min. Absorbance was monitored at 344 nm. Metabolites were identified by comparison with retention times of authentic standards. The amount of each tetraol was determined by comparison with a standard curve that had been generated from the absorption areas of authentic tetraoles analyzed in parallel under identical assay and extraction conditions using the same internal standard.

Separation of all 7,8-diol-B[a]P oxidation products could be well achieved under these conditions. In all cases, CYP1A1 produced DE1 and DE2, which finally hydrolyzed nonenzymatically to the four tetraol metabolites (33). In an additional analysis, the total rates of tetraol 3 + tetraol 4, representative for DE1 formation, and tetraol 1 + tetraol 2, representative for DE2 formation, were calculated. The DE2-derived metabolites were formed at a clearly higher rate compared with DE1-derived metabolites. The DE2:DE1 ratio was ~2-3 and did not depend on the inhibitor concentration (data not shown). Formation rates were determined for both metabolites DE1 and DE2; however, kinetic analyses were performed only with the DE2 product because it is both the major and the ultimate carcinogenic product.

AHH and EROD Assay. Hydroxylation of B[a]P to 3-OH-B[a]P was detected and quantitated with fluorescence spectroscopy (34). Preparation of the reconstituted system, preincubation, and reactions were performed as described above for the epoxidation assay, except that 20 pmol of CYP1A1 were taken per assay.

Apart from the use of a reconstituted CYP1A1 system, deethylation of ethoxyresorufin to resorufin was detected with fluorescence spectroscopy according to the standard EROD assay for CYP1A1 activity (35).

Preincubation Experiments. The reconstituted system containing CYP1A1, P450 reductase, and dilaurylphosphatidylcholine was preincubated

with hypericin or hyperforin in assay buffer at 37°C. At various times during preincubation, aliquots of the preincubation mixture (corresponding to 10 pmol of CYP1A1) were transferred to the reaction mixture containing substrate and NADPH. Additional reaction and analysis were performed as described above.

P450 Reductase Activity and P450 concentration. P450 reductase activity was determined as NADPH-cytochrome *c* reductase activity. Rates of NADPH-cytochrome *c* reduction were measured using an extinction coefficient of 21 $\text{mm}^{-1}\text{cm}^{-1}$ (36). One unit is defined as the amount of reductase reducing 1 μ mol of cytochrome *c* per minute at 25°C. The CYP1A1 concentration was determined by reduced CO-difference spectroscopy (37).

Data Analysis. Percentage inhibition values were plotted against log-transformed concentrations, and IC_{50} values were determined by linear regression. The mode of inhibition (*i.e.*, competitive, noncompetitive, uncompetitive, mixed type) was determined with the help of Lineweaver-Burk plots. Then, data were fitted with nonlinear regression to determine the kinetic constants, using Sigma Plot 2001 with the Enzyme Kinetics module (SPSS Science Software, Erkrath, Germany). The statistical significance of results was analyzed using one-way ANOVA (GraphPad Software, San Diego, CA).

RESULTS

Inhibition of Diolepoxide 2 Formation by St. John's Wort. In preliminary experiments, we determined the apparent K_m values for the formation of DE2 from 7,8-diol-B[a]P, for the formation of 3-OH-B[a]P from B[a]P (AHH test), and for the EROD reaction. These values were 1, 5.2, and 0.4 μ M, respectively. The following inhibition studies were performed with substrate concentrations in the order of magnitude of the K_m values or approximately two times higher (*i.e.*, with 2 μ M 7,8-diol-B[a]P, 5 μ M B[a]P, and 0.5 μ M ethoxyresorufin).

Three commercially available preparations of St. John's wort were dissolved in DMSO and checked on their capacity to inhibit diolepoxidation activity of CYP1A1. They all exhibited potent inhibition (Fig. 1). Jarsin was the most potent one; 1.3 μ g of its crude extract per milliliter inhibited diolepoxidation by 50%. IC_{50} values for Aristo and Esbericum capsules extracts were 8 and 24 μ g/ml, respectively (Table 1).

Inhibition of Diolepoxide 2 Formation by Pure St. John's Wort Constituents. With the exception of rutin, the major constituents of St. John's wort tested (hypericin, hyperforin, quercetin, rutin, and pseudohypericin; Fig. 2) turned out to be powerful inhibitors of

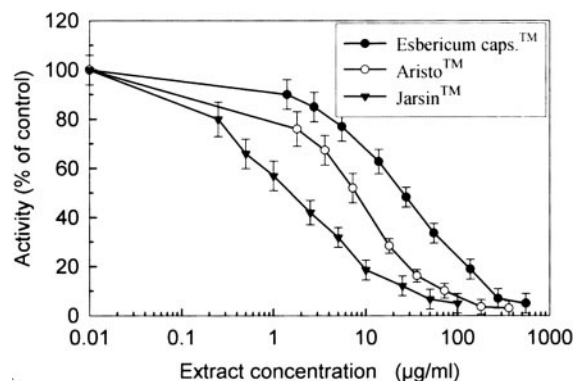


Fig. 1. Inhibition by St. John's wort preparations of 7,8-dihydrodiol-B[a]P epoxidation activity of human CYP1A1 in a reconstituted system consisting of purified human CYP1A1, purified human P450 reductase, and dilaurylphosphatidylcholine. Epoxidation activities represent rates of formation of diolepoxide 2 and were determined in the presence of the extracts as described in "Materials and Methods," with a P450 concentration of 10 nM and a substrate concentration of 2 μ M in all cases. The three commercially available extracts were Jarsin-coated tablets, Aristo drops, and Esbericum capsules (see "Materials and Methods"). Each point represents the average of duplicate determinations; bars represent SD. Statistical analysis showed that the IC_{50} values determined from the curves (see Table 1) were significantly different for Jarsin and Esbericum caps. (ANOVA, $P < 0.01$) and Aristo and Esbericum caps. ($P < 0.01$), whereas those of Jarsin and Aristo were not ($P > 0.05$).

Table 1 IC_{50} values and inhibition constants for *St. John's wort* dried extracts, and major constituents of it, on 7,8-dihydrodiol-B[a]P epoxidation activity of human CYP1A1

The epoxidation activity (formation of diolepoxide 2) was determined in a reconstituted CYP1A1 system consisting of purified human CYP1A1, purified human P450 reductase, and dilaurylphosphatidylcholine. The P450 concentration for determination of activities was 10 nM, and the substrate concentration was 2 μ M in all cases. Formation of diolepoxide 2 was determined using HPLC, as described in "Materials and Methods." Kinetic analyses were performed by first examining Lineweaver-Burk plots to determine the type of inhibition, followed by nonlinear regression to determine K_i .

Constituent	IC_{50} (μ M) ^a	K_i (μ M) ^a	Type of inhibition
Jarsin extract	1.3 \pm 0.2	3.9 \pm 0.2	Uncompetitive
Aristo extract	8.0 \pm 1.0	n.d. ^b	n.d.
Esbericum caps. extract	24.0 \pm 3.0	n.d.	n.d.
Hypericin	0.5 \pm 0.1	0.6 \pm 0.1	Noncompetitive
Pseudohypericin	8 \pm 0.6	3.2 \pm 0.8	Mixed type
Hyperforin	1.2 \pm 0.2	1.1 \pm 0.2	Competitive
Quercetin	1.5 \pm 0.2	2.4 \pm 0.8	Mixed type ^c
Rutin	~100	n.d.	n.d.

^a Inhibition experiments were performed at a substrate concentration of 2 μ M. IC_{50} values represent the mean \pm SD of three separate determinations; K_i data represent the mean \pm SE as determined by the nonlinear fit using Sigma Plot-Enzyme Kinetics software.

^b n.d., not determined.

^c The fit for mixed type inhibition was only slightly better than noncompetitive inhibition. The present experiments cannot unambiguously distinguish whether inhibition is of mixed type or noncompetitive. Nevertheless, the K_i values determined for both models were not statistically significantly different ($P > 0.05$, ANOVA).

CYP1A1-dependent epoxidation of 7,8-diol-B[a]P (Fig. 3 and Table 1). The most potent inhibitor was hypericin, with an IC_{50} value of 0.5 μ M, followed by hyperforin ($IC_{50} = 1.2 \mu$ M). Hypericin is even more potent than the well-known CYP1A1 inhibitor α -naphthoflavone, the IC_{50} value of diolepoxide 2 formation of which has been determined to be 0.8 μ M. The other constituents were also effective inhibitors but their effects were weaker than that of hypericin. To facilitate comparison of the relative inhibitory potencies of the different constituents, we plotted the reciprocal IC_{50} values (Fig. 4).

Mechanism of Inhibition. To examine the mechanism of inhibition, kinetic studies were performed with those *St. John's wort* constituents with IC_{50} values below 10 μ M (Fig. 5 and Table 1). Hypericin showed noncompetitive inhibition of CYP1A1 with K_i values of 0.6 μ M, hyperforin exhibited competitive inhibition with a K_i value of 1.1 μ M, whereas pseudohypericin and quercetin showed mixed-type inhibition with K_i values of 3.2 μ M and 2.0 μ M, respectively. An inhibition kinetics study with Jarsin extract demonstrated uncompetitive inhibition with a K_i value of 3.9 μ g/ml.

P450 reductase transfers electrons from NADPH to CYP1A1. Some chemicals may suppress reductase activity, thus blocking electron transfer and inhibiting monooxygenase activity in this way. Fig. 6 shows that only quercetin could be regarded as an inhibitor of cytochrome *c* reductase, with an IC_{50} value of 20 μ M. All of the other compounds tested in this study inhibited P450 reductase but in higher concentrations ($IC_{50} \geq 100 \mu$ M), which are irrelevant to *in vivo* conditions. Interestingly, rutin exhibited a concentration-dependent activation of cytochrome *c* reduction at concentrations above 20 μ M.

To test whether hypericin and hyperforin, the most potent inhibitors of diolepoxide 2 formation, are mechanism-based inactivators, preincubation experiments were performed. A 10-min preincubation of CYP1A1 with varying concentrations of hypericin and hyperforin in the presence of NADPH did not enhance inhibition of CYP1A1 significantly.

Inhibition of CYP1A1-catalyzed AHH and EROD Reactions. The effect of major *St. John's wort* constituents on the B[a]P hydroxylation activity of CYP1A1 has been determined with the AHH

test to measure formation of hydroxylated B[a]P products, mainly of 3-OH-B[a]P. Hypericin was found to inhibit B[a]P hydroxylation moderately with an IC_{50} value of $\sim 20 \mu$ M, whereas hyperforin exhibited significant inhibition only with concentrations above 40 μ M ($IC_{50} = 70 \mu$ M). Pseudohypericin, quercetin, and rutin did not show

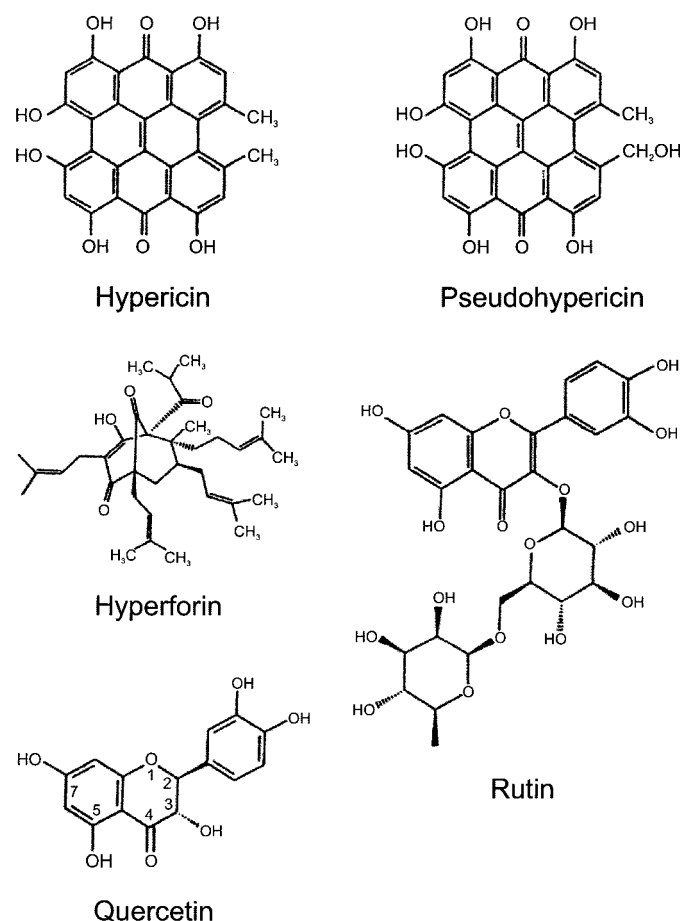


Fig. 2. Chemical structures of *St. John's wort* constituents used in the present study.

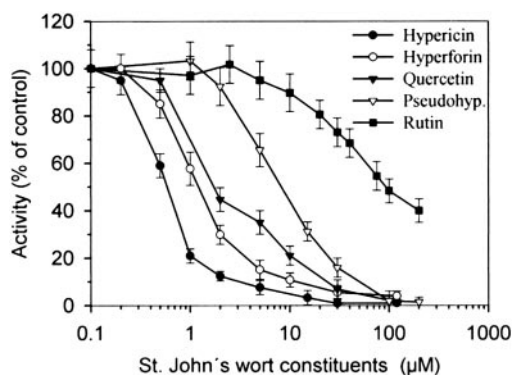


Fig. 3. Inhibition by major constituents of *St. John's wort* of 7,8-dihydrodiol-B[a]P epoxidation activity (formation of diolepoxide 2) of human CYP1A1 in a reconstituted system consisting of purified human CYP1A1, purified human P450 reductase, and dilaurylphosphatidylcholine (*Pseudohyp.*, pseudohypericin). Assays were performed with a P450 concentration of 10 nM and a substrate concentration of 2 μ M. Each point represents the mean \pm SD of three determinations. Statistical analysis exhibited that the IC_{50} values determined from these curves (see Table 1) must be considered very significantly different for hypericin and pseudohypericin, pseudohypericin and hyperforin, and pseudohypericin and quercetin (ANOVA, $P < 0.01$); significantly different for hypericin and quercetin ($P < 0.05$); and not significantly different for hyperforin and quercetin and hypericin and quercetin ($P > 0.05$).

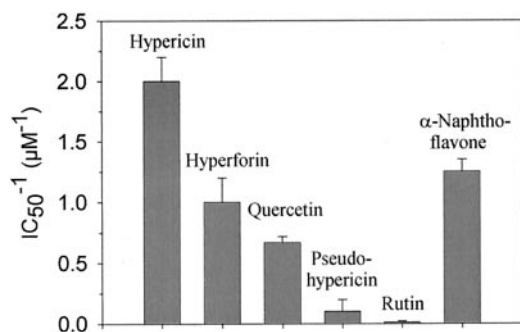


Fig. 4. Inhibitory potency of constituents of St. John's wort toward 7,8-dihydrodiol-B[a]P epoxidation by human CYP1A1. Reciprocal IC_{50} values from Table 1 were plotted for each compound. Data shown are means \pm SD of three separate determinations. For statistical analysis of the IC_{50} values, see legend to Fig. 3.

any remarkable inhibition up to $\sim 100 \mu\text{M}$ ($IC_{50} > 100 \mu\text{M}$). α -Naphthoflavone showed inhibition of B[a]P hydroxylation with an IC_{50} value of $1 \mu\text{M}$.

With regard to EROD, the conventional assay for assessment of CYP1A1 activity, quercetin exhibited a strong inhibitory potency ($IC_{50} = 0.2 \mu\text{M}$), whereas rutin showed no inhibition. Hypericin and the other St. John's wort constituents were not tested because of their intrinsic fluorescence making fluorimetric determination of the reaction product impossible. The positive control α -naphthoflavone showed inhibition of EROD with an IC_{50} value of $< 0.1 \mu\text{M}$.

DISCUSSION

We used the 7,8-diol-B[a]P epoxidation reaction to assess CYP1A1 inhibition of procarcinogen activation. This reaction is the terminal

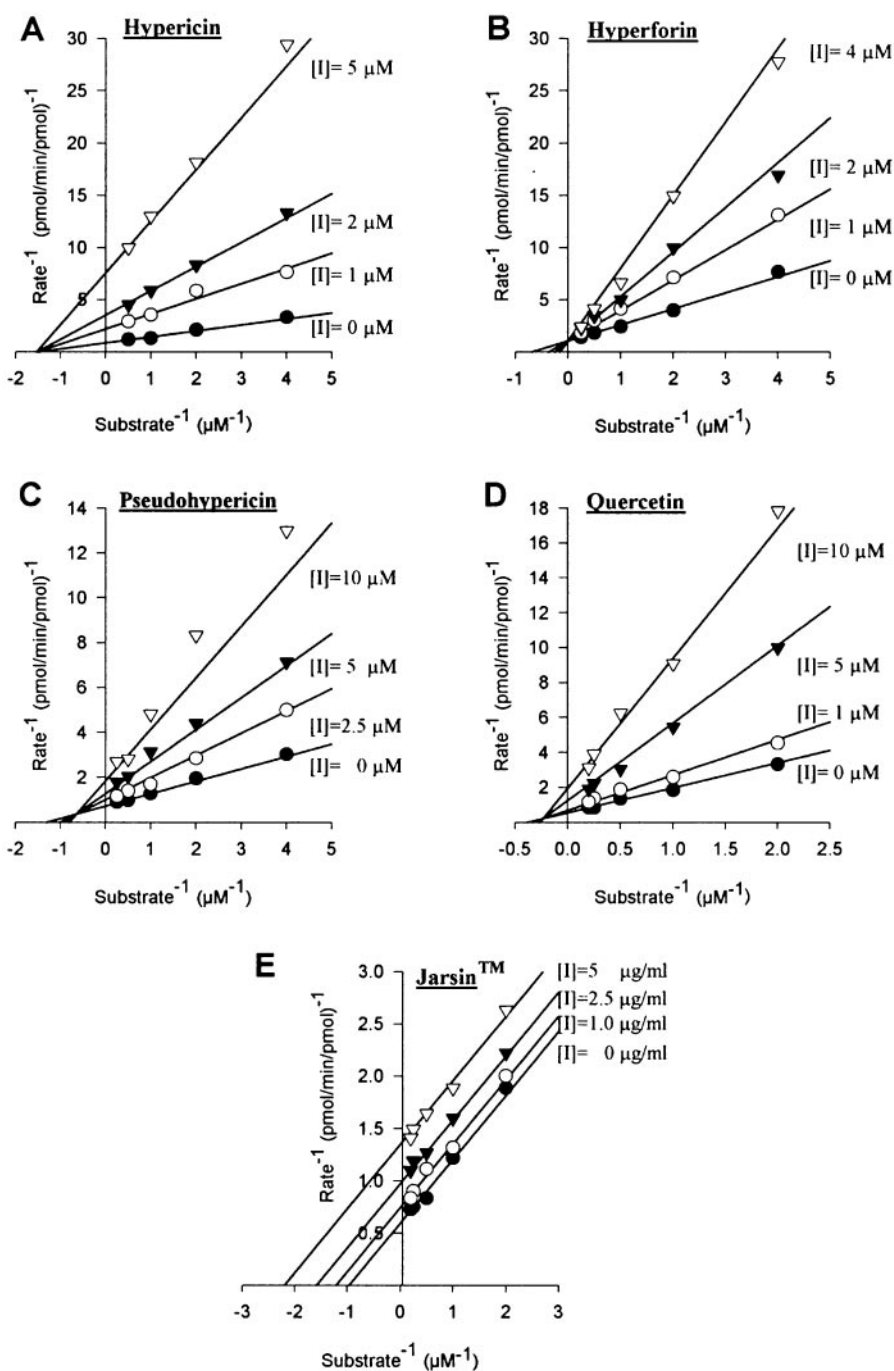


Fig. 5. Lineweaver-Burk plots of inhibition of 7,8-diol-B[a]P epoxidation activity (formation of diolepoxide 2) by constituents of St. John's wort and one St. John's wort extract. Hypericin, $0-5 \mu\text{M}$; hyperforin, $0-4 \mu\text{M}$; pseudohypericin, $0-10 \mu\text{M}$; quercetin, $0-10 \mu\text{M}$; Jarsin dried extract, $0-5 \mu\text{g/ml}$. Each point represents the average of two separate determinations. Statistical analysis exhibited that the K_i values determined from these kinetic analyses (see Table 1) must be considered very significantly different for hypericin and pseudohypericin and hyperforin and pseudohypericin ($P < 0.01$) and significantly different for hypericin and quercetin. They must not be considered significantly different for hypericin and hyperforin, pseudohypericin and quercetin, and hyperforin and quercetin ($P > 0.05$).

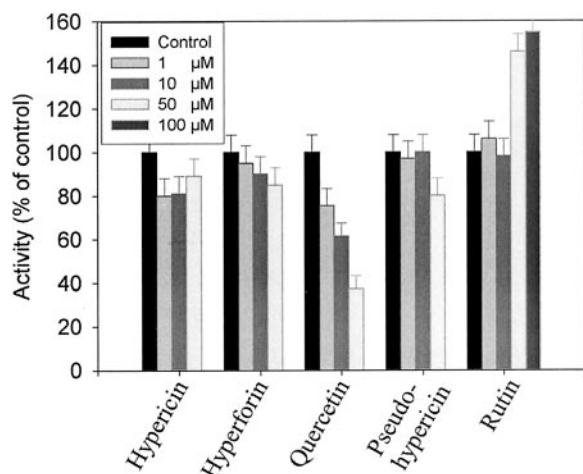


Fig. 6. Effect of St. John's wort constituents on NADPH-cytochrome *c* reductase activity in a reconstituted system containing purified NADPH cytochrome P450 reductase, cytochrome *c*, and NADPH. Each value represents the mean \pm SD of triplicate determinations. Statistical analysis showed that the activity data must be not considered quite significantly different for hypericin, hyperforin, and pseudohypericin. Quercetin exhibited a clear dose-dependent decrease in the reductase activity with significantly different values for all concentrations (ANOVA, $P < 0.05$). The data for rutin show a statistical very significant activation for concentrations $>10 \mu\text{M}$ ($P < 0.01$).

step in the process of bioactivation of procarcinogens, such as B[a]P, to the ultimate carcinogenic product, diolepoxide 2, which can bind to DNA and initiate tumorigenesis (17, 19). We recently demonstrated that, in general, the widely used EROD assay is not sufficient for the evaluation of the inhibition of carcinogen activation by human CYP1A1. In the case of resveratrol, which the EROD assay had formerly identified as a powerful, selective inhibitor of human CYP1A1 and candidate anticancer substance (23), we demonstrated that it only slightly inhibited diolepoxidation of 7,8-diol-B[a]P and did not affect B[a]P hydroxylation in the AHH test (28).

St. John's wort preparations were shown to potently inhibit the formation of diolepoxide 2 by human CYP1A1. Comparison of our results with those in published literature on other P450 activities (10) demonstrated that CYP1A1 epoxidation is the most St. John's wort-sensitive activity among them all. In the latter study, CYP2D6 was found most sensitive to inhibition, with 50% inhibition of activity exhibited by $9.1 \mu\text{g/ml}$ of extract/milliliter of incubation. IC_{50} values for the inhibition of diclofenac-4'-hydroxylation by CYP2C9 and testosterone 6 β -hydroxylation by CYP3A4 were 19 and $40 \mu\text{g/ml}$, respectively. Other P450 activities were less sensitive.

Among the constituents of St. John's wort tested, hypericin and hyperforin were found to be the most potent inhibitors of CYP1A1 epoxidation activity, with IC_{50} values of 0.5 and $1.2 \mu\text{M}$, respectively. CYP1A1 turned out to be the most sensitive of the five major human liver P450 enzymes involved in xenobiotic metabolism. Only I3,II8-biapigenin, a biflavone occurring exclusively in the buds and blossoms of *Hypericum perforatum*, exhibited a greater inhibitory capacity with an IC_{50} value of $0.082 \mu\text{M}$ for CYP3A4-catalyzed 6 β -hydroxylation of testosterone (10). It remains to be examined how the biflavone affects DE2 formation from 7,8-diol-B[a]P.

For the observed inhibition of CYP1A1 by a whole crude St. John's wort preparations, it would be difficult to determine which constituent(s) is responsible for the CYP1A1 inhibition. Hypericin and hyperforin were found the most potent inhibitors. Thus, it could be suggested that these constituents would most likely be responsible for the inhibition effect of the whole extract. However, besides the inhibitory potency, in the case of such a complex mixture of constituents, the relative abundance of each compound in the preparation would also have an impact on the contribution of a distinct compound

to inhibition, and other constituents of St. John's wort not studied here may contribute also. That is, the most potent inhibitor may be present in the extract at a much lower quantity than a less potent inhibitor.

As mentioned previously, there are no data for direct comparison of our results with regard to epoxidation activity of CYP1A1. However, based on EROD activity, two stilbene derivatives, rhapontigenin and tetramethoxy-stilbene, were reported to belong to the most potent CYP1A1 inhibitors known to date (26, 27). In particular, tetramethoxy-stilbene, a stilbene analogue designed to inhibit estradiol activation by CYP1B1, exhibited potent inhibition of CYP1A1 with an IC_{50} value of $0.3 \mu\text{M}$ for EROD. Because these results were obtained using bacterial membranes and not purified CYP1A1, a direct comparison with our findings is not possible. Moreover, EROD inhibition cannot be assessed with hypericin because of the very high intrinsic fluorescence of this substance. In any case, hypericin is a comparably potent inhibitor of CYP1A1 based on epoxidation activity, but it remains to be evaluated whether rhapontigenin and tetramethoxy-stilbene are potent inhibitors of CYP1A1 epoxidation activity, too.

A good inhibitor of CYP1A1-mediated carcinogen activation should effectively inhibit epoxidation of 7,8-diol-B[a]P but should not affect hydroxylation of B[a]P, which is the first step toward detoxification. We demonstrated that even hypericin showed only a slight influence on the B[a]P hydroxylation activity in the lower micromolar range. All of the other St. John's wort constituents practically do not affect formation of hydroxylated B[a]P metabolites. These findings are important because 3-OH-B[a]P among the 12 isomeric phenols of B[a]P was found the most potent antagonist of DE2 mutagenicity, possibly by inhibiting of 7,8-diol-B[a]P activation (38).

The inhibition of diolepoxidation may occur at either the terminal CYP1A1 enzyme or at the P450 reductase level. With the exception of quercetin, the St. John's wort constituents tested did not influence P450 reductase activity significantly. Thus, the inhibition of epoxidation activity occurs at CYP1A1. The types of inhibition were different. None of the St. John's wort compounds was a mechanism-based inactivator. Hypericin showed noncompetitive inhibition whereas hyperforin was a competitive inhibitor, suggesting that it competes with the substrate 7,8-diol-B[a]P for binding site in the active center. Pseudohypericin, differing only in one additional OH group from hypericin (see Fig. 2), and quercetin exhibited mixed-type inhibition, indicating that it can compete for substrate binding with 7,8-diol-B[a]P and also may bind to a region that does not participate directly in substrate binding. The differential type of inhibition exhibited by the whole crude extract Jarsin seems to indicate that a set of compounds is responsible for the inhibitory effect of the extract and not a single component. Note that other St. John's wort constituents such as flavonoids (not studied here) may show another type of inhibition and/or a dual mechanism of inhibition with various levels of capacity at both the CYP1A1 enzyme and the P450 reductase level, as it has been reported recently for kaempferol and myricetin (11).

P450 reductase activity was only inhibited significantly by quercetin, with an IC_{50} value of $\sim 20 \mu\text{M}$. This is in accordance with a recent study (11). However, other sites of action are probably more important because at any given concentration of quercetin, epoxidation activity of CYP1A1 is inhibited to a greater extent than is cytochrome *c* reductase activity. Other St. John's wort constituents may interfere with the activities of the CYP1A1 enzyme or of P450 reductase at different concentration levels, as it has been reported recently for kaempferol and myricetin (11), or may exhibit a concentration-dependent activation of P450 reductase as the data for rutin showed. It remains to be elucidated whether this property is exhibited by all flavanols carrying a rutinoid substituent in 3-position (Fig. 2) and possibly other polyphenols. A similar stimulation of reductase activity was observed for (-)-epigallo catechin gallate, a major tea polyphenol.

nol with a basic structure similar to flavanols and a gallate substituent in 3-position as rutin.²

St. John's wort preparations are complex mixtures of a huge variety of compounds. The concentrations of the various constituents and their inhibitory potencies are different; therefore, it is difficult to single out the main inhibitor of CYP1A1 activity under *in vitro* conditions. The *in vivo* situation of CYP1A1 inhibition is more complicated because pharmacokinetic properties (*e.g.*, distribution, bioavailability, intracellular distribution, and clearance) must be taken into account. Published data regarding pharmacokinetic distribution of St. John's wort constituents have been scarce until now. However, it is suggested that the drug is expected to come into contact with potential target tissues in which CYP1A1 is expressed and/or induced [*e.g.*, in the lung and in the gastrointestinal tract (esophagus and small intestine; Ref. 39)]. With regard to pharmacokinetic properties such as plasma concentration and clearance, hypericin, pseudohypericin, and hyperforin have been studied in healthy volunteers, and these constituents were identified as relatively low clearance drugs with half-life times of ~10 h for hypericin and pseudohypericin and 16 h for hyperforin (40). Their plasma concentrations can reach steady-state concentrations ~10 µg/liter (corresponding to 0.02 µM; hypericin and pseudohypericin) and 1.5 mg/liter (corresponding to 2.8 µM; hyperforin). With regard to the intracellular concentration, it is likely that hypericin as a highly hydrophobic substance is predominantly localized in the different cellular membrane systems (41). Indeed, for hypericin, localization experiments in human glioblastoma and carcinoma cell lines proved its predominant localization at a concentration of ~1 µM in intracellular membranes, mainly the endoplasmic reticulum, the site where CYP1A1 is also localized, whereas it could not be identified in the plasma membrane (42). These data suggest that the concentration of hypericin, and probably also of other St. John's wort constituents, with the therapeutically recommended doses, can reach intracellular concentrations well above the *in vitro* IC₅₀ values determined in the present study. In this way, the hypothesis is supported that St. John's wort and/or its major constituents may be involved in the prevention of carcinogenesis in humans, by reducing the formation of ultimate carcinogens through inhibition of CYP1A1, which is known to be the major enzyme in carcinogen activation.

In conclusion, the results suggest that St. John's wort preparations and several of their major constituents such as hypericin, hyperforin, pseudohypericin, and quercetin very potently inhibit the formation of diolepoxide 2 from 7,8-diol-B[a]P, the terminal step in chemical carcinogenesis. Hypericin is one of the strongest inhibitors of CYP1A1 from natural sources ever reported; it is even stronger than the well-known selective CYP1A inhibitor α-naphthoflavone. On the basis of these data, the extracts, their constituents, and structurally related compounds should be evaluated further for cancer chemopreventive potential.

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