possible chance correlations, to give a better overall evaluation of the reliability of the result.

Conclusion

The results of the studies described show that chance correlations are a real phenomenon occurring when the number of variables screened for possible correlation is large compared to the number of observations. For this reason, some correlations are less significant than their standard p values indicate, as has been demonstrated by reference to some reported correlations.

The present study provides guidelines for the approximate incidence of chance correlations at specified r^2 values for various combinations of observations and screened variables. These data may be used prospectively in planning correlation studies. Thus, for a given lead structure around which further synthesis is being planned, the number of relevant independent variables to be considered may be related to the number of compounds planned for synthesis, so that an unacceptable risk of chance correlation will not be present. Specific correlations obtained under conditions where the guidelines indicate an appreciable risk of chance correlations should be individually checked as described.

Finally, it should be pointed out that there is no intention of advocating correlation studies only under conditions where there is a miniscule risk of chance correlations. However, the importance of having a true idea of the reliability of a correlation equation is obvious. It is one thing to develop and use a correlation equation which is known to be somewhat tenuous but quite another to believe that it has a solid foundation when, in fact, it has not.

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Supplementary Material Available: The complete version of Table I containing data from simulated correlations using random numbers (34 pages). Ordering information is given on any current masthead page.

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A Preliminary Structure–Activity Study of the Mixed-Function Oxidase Inhibitor 7,8-Benzoflavone

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A series of substituted and structural analogues of 7,8-benzoflavone were examined for their ability to inhibit benzo[*a*]pyrene oxidation by the mixed-function oxidases found in hepatic microsomes prepared from 3-methylcholanthrene- and phenobarbital-induced rats. Of all the benzoflavones tested, only 6-amino-7,8-benzoflavone possessed significant inhibitory activity toward both classes of induced mixed-function oxidases. Parameters which were found to be necessary for maximal inhibitory activity were the maintenance of an unsubstituted or specifically substituted exocyclic phenyl group on position 2, the preservation of the pyran-4-one ring, and a 6 position which is either unsubstituted or substituted with an oxidizable moiety.

7,8-Benzoflavone (α-naphthoflavone, 7,8-BF, **1**), a synthetic flavanoid, is an inhibitor of microsomal mixed-function oxidase activities.[1] These enzymes metabolize drugs, steroids, and xenobiotics, some of which are chemical carcinogens. 7,8-BF has been used for many years in mechanistic studies of the metabolic activation of carcinogens. 7,8-BF inhibits the metabolism, binding to DNA, and tumorigenesis in mouse skin of 7,12-dimethylbenz[*a*]anthracene[2] and inhibits the metabolism and carcinogenicity of 3-methylcholanthrene in mouse embryo cells[3] and mouse skin.[4] The metabolism of benzo[*a*]pyrene by hepatic microsomes isolated from rats induced with 3-methylcholanthrene,[5] 5,6-benzoflavone, and Aroclor 1254[6] is also inhibited by 7,8-BF. In contrast, 7,8-BF stimulates the metabolism of benzo[*a*]pyrene in hepatic microsomes isolated from rats induced with phenobarbital.[5] In mouse skin, 7,8-BF inhibits benzo[*a*]pyrene metabolism and binding to RNA and protein, has only a marginal inhibitory effect on benzo[*a*]pyrene binding to DNA, and has no effect on benzo[*a*]pyrene-mediated tumorigenesis.[2] Several 4′-substituted 7,8-benzoflavones have been reported to inhibit benzo[*a*]pyrene metabolism[7] but none were more potent than the parent compound.

This structure–activity study is a logical and stepwise approach toward an understanding of the mechanism of action of 7,8-BF and concentrates on the examination of three areas of the 7,8-BF molecule: the exocyclic phenyl group, the 6 position of 7,8-BF, and the pyran-4-one ring.

Results and Discussion

The inhibitory activities of the flavones and related compounds were evaluated with hepatic microsomes prepared from rats induced with 3-methylcholanthrene

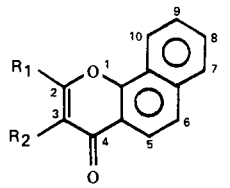
Table I. Inhibition of Benzo[a]pyrene Monooxygenase[a] by 7,8-Benzoflavones and Related Agents

no.	inhibitor	ref	I_{50},[b] μM: 3-MC microsomes[c]	I_{50},[b] μM: PB microsomes[d]
1	7,8-benzoflavone		10	>100 (21)[f]
5	3′-methoxy-7,8-benzoflavone		11	>98 (9)[f]
3	2-(2-naphthyl)-4*H*-naphtho[1,2-*b*]pyran-4-one	11	11	>100 (0)[e]
6	2′-methoxy-7,8-benzoflavone	12	13	>100 (34)[e]
7	4′-chloro-7,8-benzoflavone	13	13	>99 (0)[e]
8	6-amino-7,8-benzoflavone	14	31[g]	82
9	6-bromo-7,8-benzoflavone	15	>97 (7)[e]	>97 (0)[e]
10	4′-nitro-7,8-benzoflavone	16	>99 (0)[e]	>99 (33)[f]
2	7,8-benzoisoflavone	17	>100 (18)[e]	>100 (6)[f]
11	3′-nitro-7,8-benzoflavone	16	>100 (36)[e]	>100 (42)[f]
12	4′-methoxy-7,8-benzoflavone	13	>100 (46)[e]	>100 (0)[e]
4	2-phenyl-4*H*-naphtho[1,2-*b*]furan	18	>104 (11)[e]	>104 (34)[e]
13	6-nitro-7,8-benzoflavone	19	>200 (34)[f]	>200 (43)[f]

[a] Benzo[a]pyrene monooxygenase activity was determined as described under the Experimental Section. [b] I_{50} is the concentration of inhibitor which inhibits the enzymatic reaction by 50% and is expressed in μM for three replicate samples: Benzo[a]pyrene concentration was 60 μM and the specific activity of untreated controls in 3-methylcholanthrene-induced microsomes was 2.1–3.5 and in phenobarbital-induced microsomes was 0.80–1.02 nmol of benzo[a]pyrene oxidized/min^{-1} (mg of protein)$^{-1}$. Protein concentration in the assay was: 3-methylcholanthrene-induced microsomes, 0.200 mg/mL; phenobarbital-induced microsomes, 0.400 mg/mL. The coefficient of variation ranged from 10 to 25%. [c] 3-MC microsomes are hepatic microsomes isolated from male Sprague–Dawley rats pretreated with 3-methylcholanthrene (see Experimental Section). [d] PB microsomes are hepatic microsomes isolated from male Sprague–Dawley rats pretreated with phenobarbital (see Experimental Section). [e] Percent inhibition of benzo[a]pyrene monooxygenase at the stated concentration. [f] Percent stimulation of benzo[a]pyrene monooxygenase at the stated concentration. [g] Significantly different than 1, 3, and 5–7 at the $p = 0.01$ level.

and phenobarbital using the benzo[a]pyrene monooxygenase assay,[8] a procedure which measures the overall metabolism of benzo[a]pyrene. 7,8-BF exhibited an I_{50} of 10 μM with 3-methylcholanthrene-induced (cytochrome P-448) microsomes and had no inhibitory effect up to 100 μM with phenobarbital-induced (cytochrome P-450) microsomes (Table I).

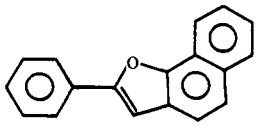
Substitution on the exocyclic phenyl ring at position 4′ with chlorine (**7**) had virtually no effect on the inhibitory activity of the flavone in microsomes prepared from 3-methylcholanthrene-induced rat liver. Other 4′-substituted 7,8-BF (i.e., 4′-fluoro, 4′-bromo, 4′-hydroxy, and 4′-hydroxy-3′-methoxy) have been tested as inhibitors of aryl hydrocarbon hydroxylase, an enzyme system similar to benzo[a]pyrene monoxygenase. All of these flavones were found to be similarly effective as 7,8-BF at equimolar concentrations in 3-methylcholanthrene-induced microsomes.[7] However, substitution at position 4′ with methoxy (**12**), nitro (**10**) and benzyloxy[7] caused a significant loss in inhibitory activity. Substitution at position 3′ with methoxy (**5**) had no effect, while nitro (**11**) substitution markedly reduced activity. Substitution at position 2′ with methoxy (**6**) or replacement of phenyl with a naphthyl group (**3**) did not affect inhibitory activity.



1 R_1 = 4′-phenyl R_2 = H

2 R_1 = H R_2 = PHENYL

3 R_1 = 2-NAPHTHALENYL R_2 = H



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These results do not clearly indicate whether steric or electronic (or both) influences are operative at the exocyclic phenyl group of 7,8-BF. The opposite effects obtained with methoxy and nitro substitution at position 3′ may be the result of repulsive ionic forces between nitro and some amino acid and not the ability to donate or withdraw electron density from the phenyl ring. Although both 3′-nitro- (**11**) and 4′-nitro-7,8-BF (**10**) did not exhibit an I_{50} of less than 99 μM, **11** did significantly inhibit enzymatic activity at that concentration (Table I). The marked differences observed with the three methoxy-7,8-BF (**5**, **6**, and **12**) and the results reported for 4′-hydroxy- and 4′-(benzyloxy)-7,8-BF lead to the conclusion that the portion of the cytochrome P-448 which binds the phenyl ring seems to have rigid spacial requirements.

Substitution at position 6 on the naphthalene ring (**9** and **13**) also resulted in a complete loss of activity of the flavone when these substituents were nonoxidizable. With an amino group at position 6 (**8**), inhibitory activity was moderately reduced in 3-methylcholanthrene-induced microsomes and, surprisingly, was observed in phenobarbital-induced microsomes, the first reported example of a 7,8-BF inhibiting that type of induced microsome. It is interesting to note that **3** and **6** were weak inhibitors of enzymatic activity from phenobarbital-induced microsomes, while all the other flavones examined were inactive. Finally, contraction of the pyran-4-one ring to a furan (loss of the carbonyl group) (**4**) or substitution of the phenyl ring on the 3 position (**2**) resulted in complete loss of inhibitory activity.

The stimulation of enzymatic activity by **1** and other flavones has been reported earlier.[5,7] The nitroflavones **10** and **11** stimulate the enzyme activity in phenobarbital-induced microsomes, and **13** stimulates the activity in both types of induced microsomes. At present, the nature of this stimulation is not clearly understood.

7,8-BF is a competitive inhibitor of benzo[a]pyrene oxidation, as measured by the conversion of the substrate to hydroxylated benzo[a]pyrenes.[9] Estabrook concluded that the first step of the multistage mixed-function oxidation pathway is the binding of the substrate to the oxidized cytochrome P-450.[10] The specificity of 7,8-BF with regard to 3-methylcholanthrene-induced microsomes

may be due in part to its ability to bind selectively to those cytochromes. Alternatively, 7,8-BF might itself require preliminary oxidation (metabolic activation) by the mixed-function oxidases in order to exert its inhibitory action. A possible site of oxidation may be position 6. The results obtained from substitution at position 6 could be interpreted to support this argument. The results observed with **8**, especially the activity found in phenobarbital-induced microsomes, suggest that the specificity observed with 7,8-BF and 3-methylcholanthrene-induced microsomes may be related to the ability of those microsomes to oxidize at carbon-6. Conversely, phenobarbital-induced microsomes may be unable to metabolize directly at that position.

The results described here indicate that whatever the mechanism of action of 7,8-BF, certain structural requirements of the flavone are necessary for maximal inhibitory activity. These are the maintenance of an unsubstituted or specifically substituted exocyclic phenyl group on position 2, the preservation of the pyran-4-one ring structure, and a 6 position which is either unsubstituted or substituted with an oxidizable moiety.

Experimental Section

All but one of the flavones and related agents were either purchased commercially or synthesized according to literature procedures. Purification was performed by repetitive recrystallization or column chromatography on silica gel until the melting point equaled or exceeded that found in the literature. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Ultraviolet spectra were recorded on a Beckman spectrophotometer, Model 25. Infrared spectra were recorded on a Beckman Acculab spectrophotometer. Elemental analyses were performed by the Environmental Support Research Laboratory, Research Triangle Park, N.C.

2-Acetyl-1-naphthalenyl 3-Methoxybenzoate. 3-Methoxybenzoyl chloride was prepared in situ by refluxing 3-methoxybenzoic acid (7.6 g, 50 mmol) with oxalyl chloride (4.3 mL, 6.4 g, 50 mmol) and 100 mL of dry benzene for 1 h. After cooling, the volatile materials were removed by evaporation under reduced pressure, and the crude solid acid chloride was refluxed with 2-acetyl-1-naphthol (9.3 g, 50 mmol) and 75 mL of dry pyridine for 4 h. At the completion of the reaction, the pyridine was removed by evaporation at reduced pressure, the residue was dissolved in chloroform and extracted several times with 0.1 N NaOH and then with 0.1 N HCl, and the chloroform solution was dried over anhydrous $CaCl_2$. After filtration and evaporation, the residue was recrystallized in methanol–chloroform to yield 9.77 g (61%) of the ester as colorless plates: mp 104.3–105.8 °C; IR (KBr) ν 1745, 1685 cm^{-1}. Anal. ($C_{20}H_{16}O_4$) C, H.

1-(1-Hydroxy-2-naphthalenyl)-3-(3-methoxyphenyl)-1,3-propanedione. The previous ester (4.0 g, 12.5 mmol) was rearranged by refluxing with KOH (800 mg, 14.3 mmol) in 40 mL of dry pyridine for 3 h.[16] After cooling, the reaction mixture was diluted with water and acidified with glacial acetic acid to pH 6.0. The crude product was filtered, washed thoroughly with water, dried, and recrystallized from ethanol to yield 2.2 g (55%) of the diketone as yellow crystals: mp 107–109.5 °C; IR (KBr) ν 1625, 1595 cm^{-1}. Anal. ($C_{20}H_{16}O_4 \cdot 0.5H_2O$) C, H.

3′-Methoxy-7,8-benzoflavone (5). The diketone (2.2 g, 6.88 mmol) was cyclized by refluxing for 90 min in 98 mL of ethanol with 22 mL of concentrated H_2SO_4. After cooling, the reaction mixture was evaporated to one-half volume. Water was added to precipitate the product, which was filtered, dried over P_2O_5, and recrystallized from ethanol to yield 1.67 g (76%) of **5** as colorless needles: mp 186.5–188.5 °C; UV (acetonitrile) λ_{max} 277 nm (ϵ 32 400); IR (KBr) ν 1655, 1635 (sh), 1615 cm^{-1}. Anal. ($C_{20}H_{14}O_3 \cdot 0.5H_2O$) C, H.

Preparation of Induced Microsomes. Male Sprague–Dawley rats (60–80 g) were maintained on lab chow and water ad libitum. Groups of at least four rats were induced either with phenobarbital sodium (Mallinkrodt Chemical Co., St. Louis, Mo.), 80 mg/kg in 0.5 mL of saline injected ip on each of 3 successive days, or with 3-methylcholanthrene (Sigma Chemical Co., St. Louis, Mo.), 20 mg/kg in 0.5 mL of corn oil injected ip on each of 2 successive days. The rats were starved for 24 h after the last treatment, and microsomes were prepared according to the method of Van der Hoeven et al.[20] Protein concentrations were determined by the method of Lowry et al.[21] using bovine serum albumin as protein standard (Sigma Chemical Co., St. Louis, Mo.).

Benzo[*a*]pyrene Monooxygenase Assay. Benzo[*a*]pyrene monooxygenase was assayed by the method of Nesnow et al.[8] as follows: A 1.0-mL incubation mixture contained microsomal protein; 1 μmol of NADP; 4.5 μmol of glucose 6-phosphate; 1.80 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.); 3 μmol of $MgCl_2$; 50 μmol of potassium phosphate buffer, pH 7.50; and 60 nmol of [^{3}H]benzo[*a*]pyrene (Amersham-Searle Co., Arlington Heights, Ill.), specific activity 12–25 μCi/μmol, with and without inhibitor as appropriate. Inhibitor was dissolved in 25 μL of acetone (except for **10** and **11**, which were dissolved in dimethylformamide). Reaction was begun upon addition of substrate dissolved in 50 μL of acetone–methanol (1:2) and, after 15 min of incubation at 37 °C, was terminated by the addition of 1.0 mL of 0.5 N NaOH in 80% aqueous ethanol. Each sample was vortexed, 3.0 mL of spectrograde hexane was added, and the sample was vortexed again for 1.5 min. After centrifugation for 10 min at 2500 rpm, 10 μL of trifluoroacetic acid was added carefully to the hexane phase, and, after 2 min, the assay mixture was vortexed and recentrifuged. An aliquot (300 μL) of the lower phase was removed and neutralized with 0.5 N HCl, and the radioactivity was determined by liquid scintillation spectrometry. Each sample was corrected for background by subtracting the activity obtained using ethanolic NaOH-treated microsomes in the incubation mixture. Control incubations contained microsomes, NADPH generating system, buffer, [^{3}H]benzo[*a*]pyrene, and 25 μL of the solvent in which the inhibitor was dissolved. At the concentrations used, the solvents, acetone and dimethylformamide, had no effect on benzo[*a*]pyrene monooxygenase from 3-methylcholanthrene-induced microsomes. The enzymatic activity from phenobarbital-induced microsomes was inhibited by acetone and dimethylformamide at 33 and 44%, respectively. Microsomal protein concentration was 0.400 mg/mL using phenobarbital-induced microsomes and 0.200 mg/mL with 3-methylcholanthrene-induced microsomes. It has been determined that in assays employing phenobarbital-induced rat liver microsomes 91% of the total benzo[*a*]pyrene metabolites formed are measured, while, with 3-methylcholanthrene-induced rat liver microsomes, 87% of the total benzo[*a*]pyrene metabolites formed are measured. The concentration of inhibitor which causes a 50% reduction in enzymatic activity (I_{50}) was determined for each inhibitor by constructing a dose–response relationship between log concentration of inhibitor and percent enzyme activity. At least four concentrations of inhibitor were examined for each agent.

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Folate Antagonists. 15. 2,4-Diamino-6-(2-naphthylsulfonyl)quinazoline and Related 2,4-Diamino-6-[(phenyl and naphthyl)sulfinyl and sulfonyl]quinazolines, a Potent New Class of Antimetabolites with Phenomenal Antimalarial Activity[1,2]

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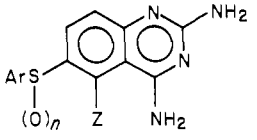
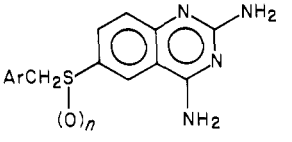
Oxidation of an array of 2,4-diamino-6-(arylthio)quinazolines provided the corresponding arylsulfinyl and arylsulfonyl analogues. A variety of these nonclassical analogues of methotrexate exhibited suppressive antimalarial activity superior to that of the parent thioquinazolines against drug-sensitive lines of *Plasmodium berghei* in mice and *P. gallinaceum* in chicks, and several displayed potent prophylactic activity against *P. gallinaceum*. The sulfinyl- and sulfonylquinazolines also retained antimalarial effects against chloroquine-, cycloguanil-, and DDS-resistant lines of *P. berghei* in mice and against chloroquine- and pyrimethamine-resistant strains of *P. falciparum* in owl monkeys. Coadministration of one of the most active of these compounds, 2,4-diamino-6-(2-naphthylsulfonyl)-quinazoline (**35**), with sulfadiazine to monkeys infected with *P. falciparum* or *P. vivax* led to greatly enhanced activity and prevented the development of quinazoline resistance.

We have recently described the synthesis and extraordinary antimalarial and antibacterial effects of a group of 2,4-diamino-6-[(phenyl and naphthyl)thio]-quinazolines Ia.[4,5] Oxidation of the related 2,4-diamino-6-[(aralkyl)thio]quinazolines IIa to the corresponding sulfoxides IIb and sulfones IIc resulted in a decrease in antimalarial activity.[6] It was deemed of interest, however, to examine the effect of oxidation on the dramatically more potent 2,4-diamino-6-(arylthio)-quinazolines Ia. The successful outcome of these studies is presented in this article.



Ia, $n = 0$
b, $n = 1$
c, $n = 2$



IIa, $n = 0$
b, $n = 1$
c, $n = 2$

Ar = phenyl or naphthyl

Chemistry. The 2,4-diamino-6-[(phenyl and naphthyl)thio]quinazolines Ia, prepared as described previously,[5] when treated either with the bromine complex of 1,4-diazabicyclo[2.2.2]octane[7] in 70% aqueous acetic acid or with a slight excess of 30% hydrogen peroxide in acetic acid afforded the corresponding sulfoxides Ib (Table I; 1–18).

The sulfones Ic (Table II; **19–37**) were obtained from the parent (arylthio)quinazolines Ia by treatment with a large excess of 30% hydrogen peroxide in glacial acetic acid. Generally, these procedures were straightforward, and the products could be isolated and purified relatively easily. The oxidation of 2,4-diamino-5-chloro-6-[(*o*-chlorophenyl)thio]quinazoline to the corresponding sulfone (**20**; Table II) was more complex, leading to a mixture which included the sulfoxide and the desired sulfone. Fractional crystallization allowed the isolation of the sulfone **20** in only 10% yield, and an attempt to repeat the procedure led only to an inseparable mixture.

Parenteral Suppressive Antimalarial Screening in Mice. The sulfoxides Ib (1–18; Table I) and sulfones Ic (**19–37**; Table II) described herein were evaluated initially against a normal drug-sensitive strain of *Plasmodium berghei* in mice by the parenteral route.[8,9] The compounds were dissolved or suspended in sesame or peanut oil and were administered to mice in a single subcutaneous dose 72 h after infection. Compounds are arbitrarily considered to be "active" when they produce at least a 100% increase in the mean survival time of treated mice, and the animals are considered to be "cured" if they survive 60 days postinfection. Results are summarized in Tables III and IV. For comparison, the minimum curative dose (MCD) for the corresponding unoxidized compounds Ia is included, as are data for two reference drugs, cycloguanil hydrochloride and pyrimethamine.

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