In vivo Activation of Zoxazolamine Metabolism by Flavone

Abstract. *The metabolism of zoxazolamine to 6-hydroxyzoxazolamine by liver microsomes from neonatal rats is stimulated severalfold by the in vitro addition of flavone, a naturally occurring compound found in several plant species. The intraperitoneal injection of flavone into neonatal rats causes an immediate severalfold stimulation in the rate of total body metabolism of simultaneously administered zoxazolamine. This is the first demonstration of stimulation of oxidative drug metabolism in vivo by a xenobiotic that is an activator of hepatic microsomal monooxygenases in vitro.*

Treatment of animals with many drugs and other foreign chemicals induces the synthesis of hepatic cytochrome P-450–dependent monooxygenases that metabolize drugs, environmental pollutants, and various endogenous substrates (*1*). This increase in the concentration of monooxygenases is important pharmacologically, for it leads to an accelerated biotransformation of xenobiotics and normal body substrates in vivo and causes an altered action of these chemicals. At least several hours is required for the induction of measurable increases in the concentration of hepatic monooxygenases (*1*). More recently, it has been found that the oxidation of various drugs and chemical carcinogens by hepatic cytochrome P-450–dependent monooxygenases can be stimulated in vitro by the addition of certain foreign chemicals to the incubation medium. This activation process, in contrast to enzyme induction, does not depend on the synthesis of new enzyme protein. Examples of in vitro activators of xenobiotic oxidations include ethyl isocyanide and acetone which stimulate aniline hydroxylation (*2*), sodium cholate which stimulates benzphetamine *N*-demethylation (*3*), betamethasone which stimulates the 2-hydroxylation of biphenyl (*4*), metyrapone which stimulates acetanilide hydroxylation (*5*), and 7,8-benzoflavone which stimulates benzo[*a*]pyrene hydroxylation (*6*). The addition of 7,8-benzoflavone or flavone to a monooxygenase system derived from human liver increases by severalfold the rate of hydroxylation of antipyrine, benzo[*a*]pyrene, and zoxazolamine (*7*), as well as the metabolism of aflatoxin B_1, benzo[*a*]pyrene 7,8-dihydrodiol and benzo[*e*]pyrene 9,10-dihydrodiol to mutagenic products (*8, 9*). The possibility that in vitro activators of microsomal monooxygenases can stimulate the in vivo metabolism of drugs has been explored in our laboratory, and we report here the first example of rapid in vivo activation of the oxidative metabolism of a foreign chemical. In this study, we found that administration of flavone to neonatal rats causes an immediate stimulation in the total body metabolism of zoxazolamine.

Zoxazolamine is a centrally acting muscle relaxant (*10*) that is metabolized to 6-hydroxyzoxazolamine by cytochrome P-450–dependent monooxygenases in liver microsomes (*11, 12*). Although zoxazolamine is no longer used therapeutically, it is an excellent model compound for studies on factors influencing drug oxidations. We have utilized a radiometric assay which provides a simple and extremely sensitive measurement of zoxazolamine hydroxylation (*12*). The principle of the assay is based upon measurement of tritium released as ^3H_2O from the 6-position of [4,6-^{3}H]-zoxazolamine during the hydroxylation reaction.

[4,6-^{3}H]Zoxazolamine was prepared as previously described (*12*). Neonatal CD rats (4 to 5 days old, 9.8 ± 0.2 g) were obtained from Charles River, Wilmington, Massachusetts. The animals were injected intraperitoneally with 740 nmole of [4,6-^{3}H]zoxazolamine (2.5 μCi, 3.4 μCi/μmole) in 20 μl of 0.02*N* HCl, immediately followed by an injection of various amounts of flavone in 40 μl of polyethylene glycol 400. Control animals received injections of zoxazolamine and polyethylene glycol 400. Animals were homogenized in five volumes of ice-cold water using a Waring blender at 15, 30, 60, or 120 minutes postinjection. Zero-time control animals were killed immediately following the injections. After rapid filtration of the homogenate through cheesecloth, a 2-ml portion of the filtrate was added to an equal volume of 10 percent trichloroacetic acid and the sample was blended with a Vortex mixer and placed in an ice bath. One-half gram of alkaline activated charcoal (Norit A) was added to absorb unmetabolized zoxazolamine as well as 6-hydroxyzoxazolamine. The sample was again blended with a Vortex mixer, centrifuged at 2300*g* for 10 minutes at room temperature, and the supernatant was passed through a disposable pipet containing a glass wool plug to remove finely suspended charcoal particles (*12*). A 1.0-ml portion of the final sample was added to 15 ml of Aquasol (New England Nuclear) and the radioactivity present as ^3H_2O was quantified by liquid scintillation spectrometry. Negligible radioactivity was found in zero-time control samples. The amount of 6-hydroxyzoxazolamine formed was calculated as described earlier (*12*).

For in vitro studies, liver microsomes from 5-day-old rats were prepared by the method of Lu and Levin (*13*). Each reaction mixture contained 3 μmole of magnesium chloride, 0.1 μmole of EDTA, 100 μmole of potassium phosphate (*p*H 7.4), 20 μmole of glucose 6-phosphate, 1 Kornberg unit of glucose-6-phosphate dehydrogenase, 0.5 μmole of NADPH, 500 nmole of [4,6-^{3}H]zoxazolamine (0.06 μCi), and various concentrations of flavone in a final volume of 0.95 ml. The reaction was initiated by the addition of

Table 1. Dose-response for the stimulation of zoxazolamine hydroxylation by flavone. For in vitro studies, flavone was added to liver microsomes obtained from 5-day-old rats. Formation of tritiated water was measured and the data are expressed as 6-hydroxyzoxazolamine. Each point represents the mean ± standard error from three determinations. For in vivo studies, rats were injected intraperitoneally with 740 nmole of [4,6-^{3}H]zoxazolamine, immediately followed by an injection of either flavone in polyethylene glycol 400 or polyethylene glycol 400 alone. The animals were killed 15 minutes later. Tritiated water in homogenates of the whole rats was measured and the data are expressed as 6-hydroxyzoxazolamine. Each point represents the mean ± standard error from four animals.

In vitro		In vivo	
Flavone concentration (μ*M*)	6-Hydroxyzoxazolamine formed (nmole/min per nmole of cytochrome P-450)	Flavone dose (μmole per rat)	6-Hydroxyzoxazolamine formed (nmole/15 min)
0	1.22 ± 0.01	0	32.4 ± 2.1
10	1.34 ± 0.02*	0.1	34.9 ± 1.9
25	1.71 ± 0.03*	0.25	38.5 ± 1.0*
50	2.14 ± 0.04*	1.0	56.3 ± 4.1*
100	2.91 ± 0.04*	2.0	77.9 ± 13.4*
250	3.45 ± 0.14*	5.0	107.1 ± 8.7*
500	3.19 ± 0.07*	10.0	98.2 ± 26.4*

*Statistically different from controls ($P < .05$) by Student's *t*-test.

0036-8075/82/0625-1419$01.00/0

50 μl of a suspension of microsomes containing 0.5 nmole of cytochrome P-450. After incubation at 37°C for 10 minutes, the reaction was terminated by addition of 0.1 ml 4*N* acetic acid. Each sample was blended with a Vortex mixer, placed in an ice bath, and 0.9 ml of water was added to bring the total volume to 2 ml. Tritiated water present in the sample was then determined, and the amount of 6-hydroxyzoxazolamine formed was calculated as previously described (*12*).

The addition of flavone to hepatic microsomes from 5-day-old rats caused a marked stimulation in the metabolism of zoxazolamine (Table 1). This activation was observed over a wide range of flavone concentrations (25 to 1000 μ*M*). A 2.8-fold stimulation in zoxazolamine metabolism was observed at a flavone concentration of 250 μ*M*.

Consistent with these results, the intraperitoneal injection of flavone into neonatal rats caused an immediate and dose-dependent stimulation in the metabolism of simultaneously administered zoxazolamine (Table 1). Although a maximum increase (3.5-fold) in the in vivo metabolism of zoxazolamine was observed with a 5-μmole dose of flavone, a significant increase was also seen with a dose of flavone as low as 0.25 μmole. This latter amount of flavone corresponds to an administered dose of 4.3 mg per kilogram of body weight.

The time course for the in vivo hydroxylation of zoxazolamine in the 5-day-old rat is illustrated in Fig. 1. Zoxazolamine was metabolized 3.5 to 5 times as rapidly when it was administered to rats along with flavone compared to when it was administered alone. This effect was observed at each time point examined and was still significant at 2 hours after the administration of flavone and zoxazolamine. After 2 hours, control animals had formed 81 nmole of 6-hydroxyzoxazolamine, representing metabolism of about 11 percent of the total dose of zoxazolamine administered. In contrast to the slow metabolism of zoxazolamine in control rats, animals coadministered 5 μmole of flavone along with zoxazolamine had formed 282 nmole of 6-hydroxyzoxazolamine at the end of 2 hours, representing metabolism of 38 percent of the total dose.

The possibility that flavone stimulated the in vivo metabolism of zoxazolamine by displacing the drug from binding sites on plasma protein or from binding sites in total body homogenates of rats was investigated in equilibrium dialysis studies. When 1 μmole of zoxazolamine per milliliter of rat plasma was dialyzed against 0.1*M* potassium phosphate buffer (*p*H 7.4) containing 0.15*M* KCl, 62 percent of the drug was bound to plasma, and this value did not change significantly in the presence of up to a 100-fold molar excess of flavone. Similar equilibrium dialysis studies provided no evidence for a displacing effect of flavone on the binding of zoxazolamine to total body homogenates of rats.

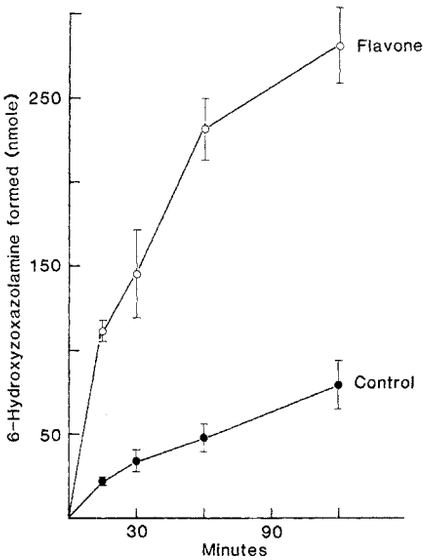

Fig. 1. Time course for the in vivo metabolism of zoxazolamine in the presence and absence of flavone. Rats were injected intraperitoneally with 740 nmole of [4,6-^{3}H]zoxazolamine, followed by an injection of 5 μmole of flavone in polyethylene glycol 400 or with polyethylene glycol 400 alone. The animals were killed at the times indicated. Tritiated water was measured and the data are expressed as 6-hydroxyzoxazolamine. Each point represents the mean ± standard error from four animals.

The data presented here demonstrate that the in vivo metabolism of zoxazolamine is immediately and dramatically increased by the concurrent administration of flavone. It is well known that when two compounds are administered simultaneously, the metabolism of one compound can inhibit metabolism of the other. However, the ability of a chemical to immediately stimulate the oxidative metabolism of a second chemical in vivo is a novel finding that may have significant pharmacological and toxicological implications.

We believe that the stimulation of in vivo drug metabolism by flavone is due to the ability of this compound to immediately increase the catalytic activities of specific cytochrome P-450–dependent monooxygenase systems in the liver. In this regard, flavone and other activators differ markedly from inducers of drug metabolism, such as phenobarbital and 3-methylcholanthrene, where stimulation of metabolism does not occur until several hours after administration and requires de novo protein synthesis (*1*). The two flavonoids thus far examined in detail, flavone and 7,8-benzoflavone, appear to activate some but not all cytochrome P-450–dependent monooxygenases. In studies with five highly purified cytochrome P-450 isozymes from rabbit liver microsomes in a reconstituted monooxygenase system, flavone was found to activate benzo[*a*]pyrene metabolism when either cytochrome P-450 LM_{3c} or cytochrome P-450 LM_4 was used as the source of hemoprotein, whereas flavone was found to be inhibitory when cytochrome P-450 LM_2, cytochrome P-450 LM_{3b}, or cytochrome P-450 LM_6 was used (*14*). Similarly, the effect of 7,8-benzoflavone on benzo[*a*]pyrene metabolism by cytochrome P-450 isozymes from rabbit liver microsomes was also found to depend on the specific form of cytochrome P-450 studied (*14*). Studies with rabbit and human liver microsomes have suggested that flavone and 7,8-benzoflavone exert their stimulatory effect, at least in part, by enhancing the interaction between cytochrome P-450 and NADPH cytochrome P-450 reductase, thereby facilitating the flow of electrons to cytochrome P-450 (*15*).

The activator used in this study, flavone, is present in man's environment as a constituent of several herbaceous plant species (*16*), and this compound is a potent activator of monooxygenases in human liver microsomes (*9*). The human diet contains a complex mixture of flavonoids, and some of these compounds activate monooxygenases in human liver microsomes whereas others inhibit these enzymes. Tangeretin and nobiletin are examples of dietary flavonoids that activate benzo[*a*]pyrene metabolism by human liver microsomes (*9*). It is not known whether human beings normally ingest enough of the activator flavonoids to stimulate the metabolism of foreign chemicals in vivo.

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Nucleotide Sequence of the Transforming Gene of Avian Myeloblastosis Virus

Abstract. *Avian myeloblastosis virus is defective in reproductive capacity, requiring a helper virus to provide the viral proteins essential for synthesis of new infectious virus. This virus arose by recombination of the nondefective helper virus and host cellular sequences present within the normal avian genome. These latter sequences are essential for leukemogenic activity. The complete nucleotide sequence of this region is reported. Within the acquired cellular sequences there is an open reading frame of 795 nucleotides starting with the initiation codon ATG (adenine, thymine, guanine) and terminating with the triplet TAG. This open reading frame could code for the putative transforming protein of 265 amino acids with a molecular weight of approximately 30,000.*

We present the complete nucleotide sequence of the transforming gene of avian myeloblastosis virus (AMV). This retrovirus is distinctive in that it causes acute myeloblastic leukemia in chickens (*1*). In vitro, AMV transforms a specific class of hematopoietic cells, but does not morphologically transform fibroblasts (*2*). Thus, it appears that only certain target cells are responsive to the AMV *onc* gene product (*2*).

The AMV provirus was isolated from a library of chicken recombinant λ phage constructed with a partial Eco RI digest of DNA from leukemic myeloblasts producing AMV (*3*, *4*). The clone (λ11A-1-1) contains the entire AMV provirus and adjacent chicken DNA sequences. Portions of the proviral genome were subcloned in the plasmid vector pBR322 and used for sequencing. One subclone contained AMV sequences located between the Kpn I site and the 3′ proximal Xba I site. The other subclone contained AMV sequences located between the 3′ proximal Eco RI site and the 3′ viral terminus as well as the adjoining cellular DNA up to an Eco RI site.

The restriction map of the cloned AMV proviral genome and the strategy employed to determine the nucleotide sequence of the Kpn I to Bgl II DNA fragment containing the transforming gene (*amv*) and the long terminal repeat (LTR) at the 3′ end of the AMV genome are shown in Fig. 1.

Within the nucleotide sequence of the 3′ end of the integrated AMV provirus (Fig. 2) we can identify the following domains: (i) the terminal portion of the polymerase gene, identified by an open reading frame extending from position 1 and terminating with a TAG (thymine, adenine, guanine) codon at position 162; (ii) a region of 350 bases without an apparent open reading frame extending between positions 165 to 515; (iii) an open reading frame of 795 bases extending from positions 516 to 1310; and (iv) the 3′ LTR adjacent to the host sequences.

Earlier studies have revealed that the AMV genome has undergone recombination in which the entire helper virus "env" gene have been replaced by cellular sequences (*4*, *5*). In order to localize the points of recombination, we have compared the carboxyl terminal sequence of the AMV polymerase gene with that of the nondefective Prague strain of Rous sarcoma virus (PR-RSV) (*6*). From position 1 to position 78 of the AMV DNA fragment in the sequence reported here, the nucleotide sequence is identical to that of PR-RSV. From position 78 to the termination signal, TAG at position 1313, the sequences of AMV and PR-RSV are entirely different, thus localizing the 5′ end of cellular insertion sequences.

The host-helper virus junction occurs at a region which constitutes a potential splice acceptor site. In general, splicing acceptor sites (at the 3′ end of intervening sequence) contain a pyrimidine-rich nucleotide tract followed by the sequence AGG. The junction point between the cellular insertion sequence and the helper viral sequence fits the consensus acceptor splice sequence (*7*).

The product of the AMV transforming gene has yet to be identified. Examination of the cellular derived *amv* sequences (Fig. 2) reveals an open reading frame starting with the initiation codon ATG at position 516 and terminating with the triplet TAG at position 1310. This stretch of 795 nucleotides could

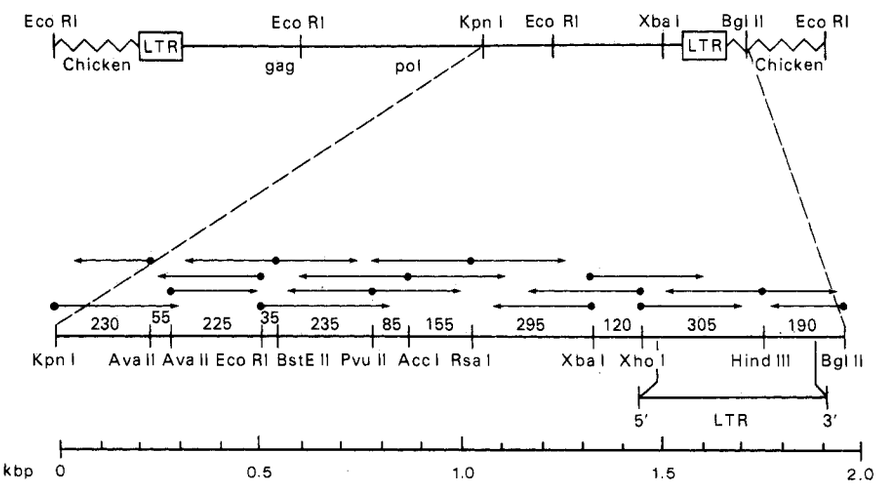

Fig. 1. Restriction enzyme map and strategy of sequencing. The Kpn I–Bgl II segment was fragmented and sequenced (*12*); the restriction sites are indicated. The 5′ ends were labeled with [γ-^{32}P]adenosine triphosphate and T4 polynucleotide kinase. The labeled end of each fragment is indicated by the closed circle. The numbers give the length of the fragments and the direction of sequencing is indicated by arrows.

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