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ENZYME INDUCTION BY COUMARINS

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ABSTRACT

The sodium pentobarbital sleeping time in mice was studied after pretreatment with coumarins (coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, acenocoumarol, bis-hydroxycoumarin, phenprocoumon and warfarin). All coumarins except bishydroxycoumarin cause a statistical significant reduction of barbiturate sleeping time, hence, have to be considered as enzyme-inducers. The largest enzyme inducing effect was observed with warfarin.

INTRODUCTION AND PURPOSE OF STUDY

The interest in coumarins is founded in an observation during the early twenties that cattle fed with decomposed sweet clover developed bleedings due to increased coagulation time (1). Smith (2) was able to find the cause of the bleeding disease: the coumarin present in sweet clover is converted by fermentation to bis-[4-hydroxy-coumarinyl-3-]methane (Dicumarol). Based on an extraction procedure by Roberts (3) Link (4) was able to isolate in 1940 the active compound in crystallized form. Since then many coumarin derivatives were synthesized. According to Fučik (5) the hydroxyl group in the 4-position seems to be of primary impor-

tance. In general substitution in the benzene ring results in decreased anticoagulant activity, whereas substitution in the pyran ring results in varying activity. Reviews on the structure-activity relationships of coumarin derivatives are found in the literature (6,7).

The coumarin anticoagulants inhibit the synthesis of vitamin K dependent clotting factors II, VII, IX and X. Many drugs can modify the anticoagulant action of coumarins by various mechanisms. These include alteration of bioavailability of vitamin K, change in absorption, distribution, protein binding, metabolism and excretion of coumarins and change in prothrombin-complex concentration by direct effect on synthesis or catabolism of clotting factors or receptor affinity for coumarins or non-prothrombin-complex-dependent hemostatic mechanisms (8). The possible sites of interaction are shown schematically in FIGURE 1 (9). Koch-Weser and Sellers (8) gave an excellent review on the present status of drug interactions with coumarins.

Coumarins are metabolized by mixed-function oxidase enzymes in hepatic microsomes (10-12). Therefore, any drug which increases or decreases the activity of the enzymes responsible for biotransformation of the coumarins may also induce or inhibit the metabolism of coumarins and their hypoprothrombinemic effect. And indeed many drugs have been reported to interact with coumarins by enzyme induction or inhibition. These and other drug interactions with coumarins have been subject of several review articles (8, 13-16). After the first reports that phenobarbital decreases bishydroxycoumarin plasma levels and anticoagulant activity (17-21) and the classical animal experiment for demonstration of this drug interaction (22) it became clear that barbiturates cause induction of hepatic microsomal enzymes

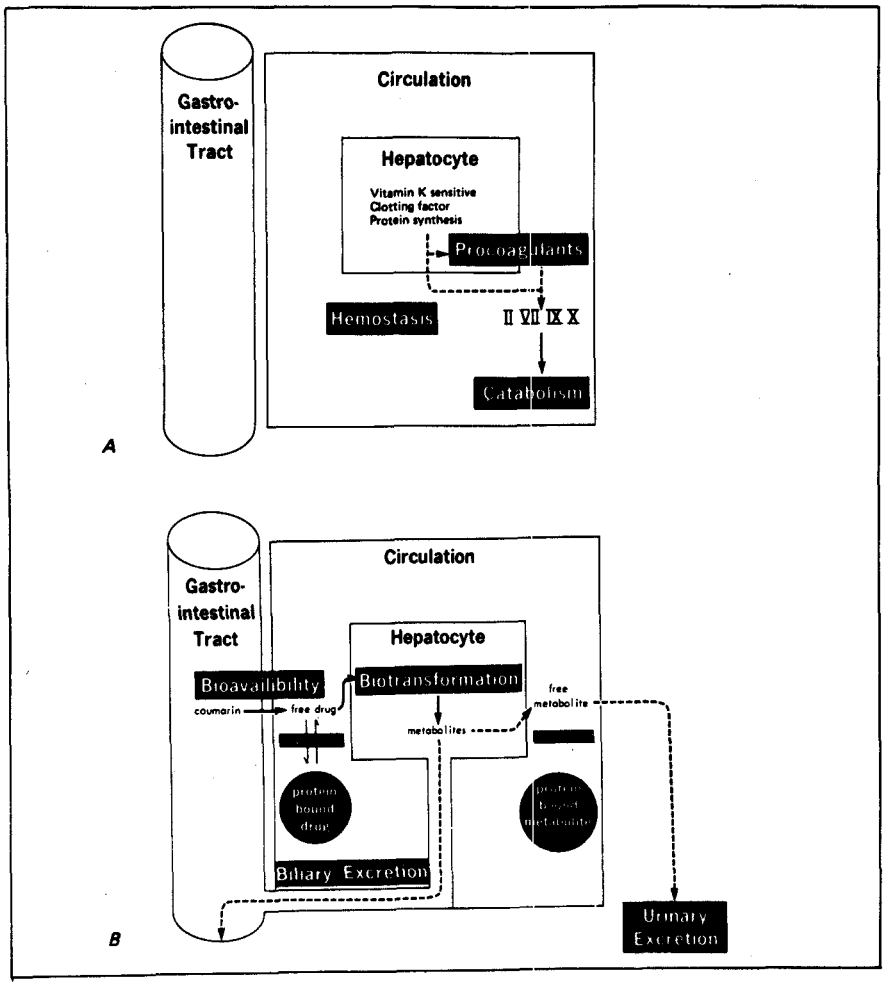

FIGURE 1: Pharmacodynamic and pharmacokinetic drug interactions with coumarin anticoagulants. 1A indicates schematically the mechanisms of pharmacodynamic interactions; i.e. pharmacological actions of coumarins which may be altered by other drugs affecting clotting factor synthesis or the haemostatic process.
1B indicates schematically the mechanisms of pharmacokinetic interactions; i.e. interactions in which absorption, biotransformation or disposition of coumarin anticoagulants may be altered. From S.M. MacLeod and E.M. Sellers, Drug 11: 461-470 (1976) (with permission from the copyright owner).

which metabolize coumarins. However, it has been stated that coumarins themselves apparently do not act as enzyme inducers (8). Based on this statement and the opposite observation in our laboratories that the pentobarbital sleeping time in mice was reduced in those animals pretreated with coumarin (1,2-benzopyrone) a study was undertaken to investigate the influence of various coumarin derivatives on the pentobarbital sleeping time as indicator of possible enzyme induction.

METABOLISM OF COUMARINS

Coumarins are metabolized by mixed-function oxidase enzymes in hepatic microsomes (10-12) resulting in various hydroxylated compounds.

Coumarin

The coumarin hydroxylating activity was found to be predominantly in the microsomal fraction (>90%) (23). The activity was low in the mitochondrial fraction (<10%) and nil in cytoplasma (23). Several compounds were identified to more or less completely inhibit enzymatic synthesis of umbelliferone from coumarin: diethyldithiocarbonate, p-chlormercuribenzoate, menadion and Na-desoxycholate (23).

The major place of hydroxylation seems to be the liver, although brain, kidney, adrenal and small intestines have low but measurable levels of drug-metabolizing enzymes which metabolize, among others, coumarin and hexobarbital (24). However, pretreatment with enzyme inducers (phenobarbital, 3-methylcholanthrene, 4-methylcoumarin) increases the hydroxylase activity only in the rat liver but not in the other tissues tested (24).

Kerékjárto (25) studied the microsomal hydroxylation of coumarin *in vitro* using liver microsomes from rats, guinea pigs and rabbits.

Schilling et al. (26) found upon P.O. administration of coumarin in man that approximately 80% of the

dose is excreted in urine as umbelliferone and between 1 to 6% as o-hydroxyphenylacetic acid, showing the difference of the major metabolic route for coumarin between man and rabbit or rat. The pharmacokinetics of coumarin and umbelliferone upon I.V. administration of coumarin in man was reported by Ritschel et al. (27). The biotransformation of coumarin to umbelliferone is extremely fast. Practically all of umbelliferone is present in blood as glucuronide (27).

Pekker and Schäfer (28) identified upon P.O. and topical administration of coumarin in rabbits in urine unchanged coumarin, umbelliferone, 4-hydroxycoumarin, o-hydroxyphenylacetic acid, 6,7-hydroxycoumarin and 3-hydroxycoumarin. Kaighen and Williams (29) found additionally o-hydroxyphenyllactic acid in urine. Williams et al. (30) found that in biliary cannulated rats approximately 50% of P.O. or I.V. administered coumarin appears in the bile as metabolites in which the heterocyclic ring has been opened. Only traces of the 7-hydroxycoumarin (0.02%) and only 5% of 7-hydroxy-coumarin glucuronide are excreted in the bile.

Mead et al. (31, 32) identified umbelliferone in animal urine after administration of coumarin and proved that coumarin derivatives are trace-metabolites of phenylalanine. Sumere et al. (33) identified umbelli-ferone in normal urine in man. Some of the coumarin derivatives seem to be potent inhibitors of enzyme hydroxylations of phenylalanine and tryptophan (34, 35). Hattori et al. (36) found that phenylketonuria, a hereditary metabolic disorder, characterized by a fail-ure of hydroxylation of phenylalanine to tyrosine in the liver, results in increased phenylalanine blood levels and umbelliferone is excreted in excessive amounts in the urine among other compounds (i.e., phenylpyruvic,

phenylacetic, phenyllactic, o-hydroxyphenylacetic acids, phenethylamine and phenylacetyl-glutamine).

Scheline (37) found that coumarin was metabolized by the rat caecal microflora to melilotic acid by ring fission.

4-Hydroxycoumarin

4-hydroxycoumarin has been identified as one of the metabolites of coumarin in rabbit urine (28). Upon administration of 4-hydroxycoumarin to rabbits and dogs the drug was primarily excreted in urine in form of conjugates with glucuronic acid and sulfuric acid, yet not as ethereal sulfate (31).

7-Hydroxycoumarin

7-Hydroxycoumarin or umbelliferone is excreted largely unchanged and as conjugates of glucuronic and/or sulfuric acid in the rat. However, upon I.P. administration in rats hydroxylation was found (38).

Upon incubation with rat caecal microflora umbelliferone is converted to 2,4-dihydroxyphenylpropionic acid (38).

Umbelliferone is the major metabolite of coumarin in man (26, 27). Most of the compound is present as glucuronide and only traces of free umbelliferone are found in plasma (27).

Acenocoumarol

Acenocoumarol is largely excreted by the kidneys in unchanged form (39, 40).

Bishydroxycoumarin

Bishydroxycoumarin is hydroxylated to inactive compounds. The metabolites and traces of the parent drug are excreted into urine (39). The extent of metabolism is more than 99% (41).

Ethyl Biscoumacetate

Approximately 15% of ethyl biscoumacetate is metabolized in the liver to the hydroxy form, which is

secreted into the bile, reabsorbed from the GI tract, then further metabolized and finally excreted into urine (42).

Phenprocoumon

Phenprocoumon is the longest acting of the peroral anticoagulants. It is apparently slowly metabolized to the hydroxy form.

Warfarin

Warfarin undergoes ring hydroxylation to an inactive metabolite 7-hydroxywarfarin and reduction of the acetonyl chain to an alcohol which gives four optically active enantiomers, some of these having anticoagulant activity (43). It is believed that the warfarin alcohols, or some of them, may have anticoagulant activity. However, it seems that high plasma levels are required. The metabolites are not affected by enzyme induction or enzyme inhibition.

DRUGS INFLUENCING BIOTRANSFORMATION OF COUMARINS

Since coumarins are metabolized by mixed-function oxidase enzymes in hepatic microsomes a rather large group of drugs have been found to either cause enzyme induction or enzyme inhibition of coumarins. Koch-Weser and Sellers (8, 13) reviewed the literature for reported observations in man. In TABLE 1 a list of drugs causing enzyme induction of coumarins in man and in TABLE 2 of drugs causing enzyme inhibition in man is given.

Conney (44) reported that the basal and phenobarbital-induced levels of a microsomal mixed-function oxidase that hydroxylates coumarin is under genetic control. 3-methylcholanthrene was not able in mice to induce coumarin hydroxylase activity. Also the levels of total microsomal cytochrome P-450 and cytochrome c reductase did not differ significantly among the different strains of mice. It is hypothesized that, if there are several types of cytochrome P-450 molecules, a pre-

TABLE 1: Drugs Actually or Possibly Causing Enzyme Induction of Coumarins in Man

Drug	Coumarins
Barbital, Amobarbital, Aprobarbital, Butabarbital, Heptabarbital, Phenobarbital, Pentobarbital, Secobarbital, Vinbarbital	Acenocoumarol, Bishydroxycoumarin, Ethyl Biscoumacetate, Phenprocoumon, Warfarin
Ethchlorvynol	Bishydroxycoumarin, Warfarin
Glutethimide	Ethyl Biscoumacetate, Warfarin
Griseofulvin	Warfarin
Meprobamate	Warfarin

TABLE 2: Drugs Actually or Possibly Causing Enzyme Inhibition of Coumarins in Man

Drug	Coumarins
Allopurinol	Bishydroxycoumarin
Chloramphenicol	Bishydroxycoumarin
Clofibrate	Bishydroxycoumarin, Warfarin
Mercaptopurine	Ethyl Biscoumacetate, Warfarin
Methylphenidate	Ethyl Biscoumacetate
Nortriptyline	Bishydroxycoumarin
Phenyramidol	Bishydroxycoumarin, Warfarin

ferential synthesis of a type with increased ability to hydroxylate coumarin might explain the differences among different strains.

Phenobarbital decreased the anticoagulant activity of bishydroxycoumarin by lowering its plasma levels in most persons studied, however, occasionally a subject did not respond to phenobarbital (17), which has been attributed to genetic variation within a species (45).

The most prominent group of drugs inducing metabolism for coumarins are the barbiturates. Reduction of coumarin activity has been reported for barbital (21,

22, 46, 47), amobarbital (18, 20, 48, 49), aprobarbital (50), butabarbital (51), heptabarbital (21, 52-54), pentobarbital (20, 47), phenobarbital (17, 18, 22, 55-63), secobarbital (18, 20, 48, 49) and vinbarbital (50).

EXPERIMENTAL

Test Preparations: Test solutions were prepared of coumarin[1], 4-hydroxycoumarin[2], and 7-hydroxycoumarin[2] with 1.5 mg/ml in 50% prophylene glycol-distilled water. The solutions were stored in a refrigerator.

Test suspensions of bishydroxycoumarin[3], acenocoumarol[4], and phenprocoumon[5] were prepared by crushing and powdering tablets in a mortar and adding distilled water q.s. to obtain a potency of 1.5 mg/ml. All suspensions were freshly prepared every day.

The warfarin sodium[6] solution was freshly prepared every day from warfarin sodium powder vials with a potency of 1.5 mg/ml.

Animals: Adult male Swiss albino mice kept under identical conditions in a climatized room with 12 hours dark-artificial light cycle on pelletized food with feeding in the morning and water ad libitum were used for the experiments.

Procedures:

Control Group: A 1.25% solution of sodium pentobarbital[7] was injected daily intraperitoneally in a

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[1]Schaper & Brümmer, Salzgitter-Ringelheim, Germany

[2]Fluka AG, Buchs, Switzerland

[3]Dicumarol 100 mg, Abbott Pharmaceuticals, Inc. North Chicago

[4]Sintrom 4 mg, Geigy Pharmaceuticals, Ardsley, N.Y.

[5]Liquamar 3 mg, Organon Inc., W. Orange, N.J.

[6]Coumadin 75 mg, Endo Laboratories Inc., Garden City, N.Y.

[7]Nembutal Sodium, Abbott Pharmaceuticals, Inc., North Chicago

dose size of 60 mg/kg. Onset and duration of sleeping time LRR (loss of righting reflex) were determined on the first and sixth day. The mice were fasted overnight prior to the first and sixth dosing.

Test Groups: The animals of the test groups received once per day for five consecutive days an intraperitoneal injection of a coumarin test preparation in a dose size of 10 mg/kg except of phenprocoumon which was given in a dose size of 2.5 mg/kg. After overnight fasting sodium pentobarbital 60 mg/kg was administered I.P. on the sixth day. For phenprocoumon the experiment was repeated with a group of non-fasting mice prior to dosing with sodium pentobarbital. Onset and duration of sleeping time (loss of righting reflex) were determined after barbiturate administration.

RESULTS AND DISCUSSION

The data for onset and duration of sleeping time and the statistical significance are listed in TABLE 3. FIGURE 2 shows the average duration of sleeping times $\pm$ S.D. of sodium pentobarbital after single and multiple dosing and after pretreatment with coumarin derivatives. As seen from TABLE 3 all coumarin derivatives except bishydroxycoumarin cause a statistical significant reduction of barbiturate sleeping time. The sleeping time after bishydroxycoumarin was slightly reduced yet not showing statistical significant difference when compared to single dose pentobarbital sleeping time, but showing a statistical significant difference when compared to the multiple dose pentobarbital sleeping time. The least yet significant reduction was found with coumarin, followed by 4-hydroxycoumarin. It is interesting to note that also acenocoumarol caused reduction in sleeping time, or apparent enzyme induction although this compound is hardly metabolized but primarily excreted in urine in unchanged form (39). The greatest reduction in sleeping time was observed with warfarin.

TABLE 3: Enzyme Induction by Coumarins in Mice After 5 Days Pretreatment with a Coumarin Compound, Na Pentobarbital was Given on the Sixth Day

Drug	Dose [mg/kg]	Average onset [min]	P*	Average duration LRR [min]	P*	No. of mice
Na-pentobarbital (1st day)	60	3.28 ±0.46	- / -	63.91 ±29.42	- / -	19
Na-pentobarbital (6th day)	60	2.77 ±0.49	0.05<P<0.025 / -	32.67 ±16.16	0.005 / -	19
Coumarin	10	3.50 ±0.67	0.5<P<0.4 / 0.025<P<0.02	42.39 ±21.02	0.01 / 0.15<P<0.10	17
4-hydroxy-coumarin	10	3.29 ±1.07	0.999<P<0.995 / 0.3<P<0.2	38.31 ±37.42	0.025<P<0.0125 / 0.35	17
7-hydroxy-coumarin	10	3.24 ±1.09	0.95<P<0.9 / 0.3<P<0.2	30.19 ±13.42	0.0005 / 0.35	18
Aceno-coumarol	10	3.61 ±1.26	0.5<P<0.4 / 0.1<P<0.05	34.31 ±27.39	0.01 / 0.45<P<0.4	10
Bishydroxy-coumarin	10	2.60 ±0.52	0.02<P<0.01 / 0.6<P<0.5	51.08 ±15.55	0.15 / 0.025	10
Phenpro-coumon	2.5	2.88 ±0.41	0.05<P<0.025 / 0.6<P<0.5	31.20 ±18.27	0.001 / 0.90	13**
Phenpro-coumon Non-Fasting	2.5	2.45 ±0.20	P<0.001 / 0.1<P<0.05	45.86 ±20.35	0.02<P<0.01 / 0.2<P<0.1	10
Warfarin sodium	10	5.07 ±3.34	0.2<P< 0.1 / 0.1<P<0.05	21.16 ±11.95	0.0005 / 0.10<P<0.05	10

* = Compared to Na-pentobarbital control
/ = against 1st day of control/against 6th day of control
** = only 13 out of 33 animals survived

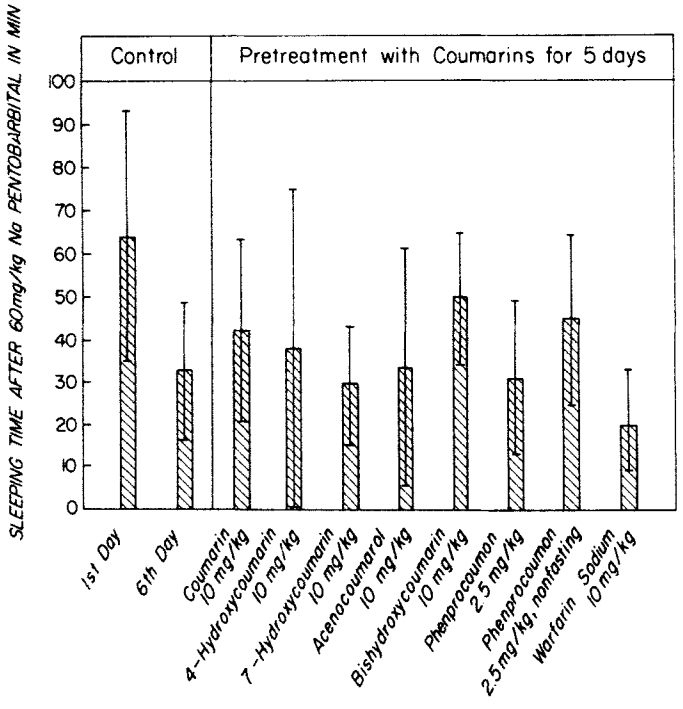

FIGURE 2: Sleeping time in mice after single and multiple dose I.P. administration of sodium pentobarbital 60 mg/kg (mean ± S.D.) and after multiple dose pretreatment with various coumarins.

In all experiments the animals survived with the dose size of 10 mg/kg of coumarin derivative for 5 days except with phenprocoumon. When the dose was reduced to 2.5 mg/kg only 13 out of 33 animals survived. However, when the experimental procedure was changed by omitting the overnight fasting all animals survived.

The onset of LRR after pretreatment with bishydroxy coumarin and phenprocoumon was statistically significant different from that of the controls after a single dose of Na pentobarbital. Except for coumarin no other

coumarin derivative had significant differences in onset of LRR when compared to that after multiple dosing with Na pentobarbital.

Although animal experiments can not necessarily be translated to human data it can be expected that barbiturate metabolism may be induced by some coumarin derivatives.

The study demonstrated that the former belief that coumarins themselves apparently do not act as enzyme inducers (8) must be revised, at least in mice.

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