Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 673–678, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0023-Z

BIOCHEMICAL BASIS OF WARFARIN AND BROMADIOLONE RESISTANCE IN THE HOUSE MOUSE, *MUS MUSCULUS DOMESTICUS*

TINA M. MISENHEIMER,* MOGENS LUND,† ANN EILEEN MILLER BAKER‡ and J. W. SUTTIE*§

*Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.; †Statens Skadedyrlaboratorium, Danish Pest Infestation Laboratory, Ministry of Agriculture, DK 2800 Lyngby, Denmark; and ‡Biology Department, Colorado State University, Fort Collins, CO 805523, U.S.A.

(*Received* 1 *May* 1993; *accepted* 20 *September* 1993)

Abstract—Danish mice (*Mus musculus domesticus*) genetically resistant to the anticoagulant action of two 4-hydroxycoumarins, warfarin and bromadiolone, were examined to determine their mechanism of resistance. The hepatic vitamin K epoxide reductase in the bromadiolone-resistant mice and in one phenotype of warfarin-resistant mice was highly insensitive to *in vitro* inhibition by warfarin and bromadiolone. The kinetic constants for the epoxide reductase from bromadiolone-resistant mice were also altered. The V_{max} for this enzyme was decreased by 40%, and the K_m for the reaction reductant, dithiothreitol, was 70% lower than that of normal mice. This phenotype of Danish resistant mice appears to have a resistance mechanism that is similar to that reported for a Welsh strain of warfarin-resistant rats. The other phenotype of Danish resistant mice had a hepatic epoxide reductase that was only slightly less sensitive to warfarin inhibition than normal. The mechanism of warfarin resistance in these mice is not apparent from the available data.

Key words: vitamin K; vitamin K epoxide reductase; 4-hydroxycoumarin; warfarin resistance; rodents

The anticoagulant warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] is used widely as a rodenticide. It acts by inhibiting the liver microsomal vitamin K 2,3-epoxide reductase and effectively blocking the production of the vitamin K-dependent plasma clotting factors. The resulting hemorrhage is lethal. The continual use of this rodenticide has resulted in the development of populations of warfarin-resistant rats [1–4] and mice [5–7] in a number of geographic locations. The biochemical basis for resistance has been shown to differ among strains of *Rattus norvegicus*. A warfarin-resistant rat strain initially trapped in Wales has a vitamin K epoxide reductase that is relatively insensitive to warfarin inhibition [8–10]. A warfarin-resistant strain initially identified in Scotland has an epoxide reductase that is as sensitive to warfarin inhibition as the normal (genetically sensitive) enzyme, but unlike the normal enzyme, the inhibition is reversible [11, 12]. A Chicago warfarin-resistant rat strain is similar to the Scottish strain in that the epoxide reductase is as sensitive to warfarin inhibition as normal, but the inhibition is only partially reversible [13]. The rate of warfarin clearance from the liver was also increased in th Chicago strain. In all three warfarin-resistant rat strains, the epoxide reductase has been altered, and this alteration is at least partially responsible for the warfarin resistance.

§ Corresponding author: Dr. J. W. Suttie, Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706. Tel. (608) 262-2247; FAX (608) 262-9338.

Rats have been shown to have a single warfarin-resistant gene that is autosomally dominant in the Welsh strain of rats [14], and autosomal with incomplete dominance in the Scottish strain of rats [15, 16]. Warfarin resistance in the house mouse has been shown to be dominant in females, but partially dominant and under the influence of modifiers in males [17]. The gene for warfarin resistance in mice is in a linkage position analogous to that in the rats [17], which suggests that the same enzyme has been affected in mice and rats. However, a recent study [18] of a warfarin-resistant mouse strain originally trapped in Cambridge, England, has not shown this alteration of the enzyme.

The increase in resistance within the wild rodent population has led to the introduction of a number of other 4-hydroxycoumarins other than warfarin as rodenticides. These include the potent anticoagulant bromadiolone (3-[α-[*p*-(*p*-bromophenyl)-β-hydroxyphenethyl]-benzyl]-4-hydroxycoumarin[19]. The objective of this investigation was to determine whether or not the hepatic epoxide reductase from mice found to be resistant to warfarin or bromadiolone differed from that of mice sensitive to these anticoagulants. If differences do exist, these assays could serve as a method for detecting resistant populations, which is often difficult because of the influence of age, sex, and diet on susceptibility [20–22].

MATERIALS AND METHODS

Animals. The livers from 22 warfarin-resistant, 20

bromadiolone-resistant, and 24 susceptible male and female mice were obtained from Mogens Lund (Danish Pest Infestation Laboratory, Denmark). The warfarin- and bromadiolone-resistant mice originated from two sites, one in the eastern part of Jutland and the other 10 km north of Copenhagen; the susceptible mice (*Mus m. musculus*) were obtained from various locations in Sealand. The warfarin-resistant mice survived the standard WHO treatment of 0.025% warfarin in the diet for 21 days. The bromadiolone-resistant mice survived a treatment of 0.01% bromadiolone in the diet for 19 days. The mice were killed 3–4 months after the termination of 4-hydroxycoumarin treatment, and the livers were removed and stored in liquid nitrogen until assay. Other female mice were trapped by Ann Baker (Colorado State University, Fort Collins, CO) on three warfarin-baited and five unbaited farms in Larimer County, CO. These farms had been baited for at least 2 years with the rodenticides warfarin, brodifacoum, or bromadiolone. These mice were held for less than a week before they were shipped. Upon arrival in Wisconsin, they were killed and livers were removed for enzyme assay without a period of storage in liquid nitrogen.

Vitamin K epoxide reductase assays. Microsomes were prepared from the thawed or fresh livers as described previously [13], and epoxide reductase activity was measured as the formation of vitamin K quinone from vitamin K 2,3-epoxide with dithiothreitol (DTT) as a reductant. *In vitro* warfarin inhibition of the epoxide reductase from the individual livers was determined by assaying liver microsomes solubilized in 0.3% sodium cholate/0.01 M *N*-tris-(hydroxymethyl)methyl-3-aminopropane sulfonic acid (TAPS)/0.25 M sucrose/0.15 M KCl (pH 8.8) ± warfarin. Assays were performed by incubating 0.20 mL solubilized microsomes in the presence of 2 mM DTT, 40 μM vitamin K epoxide (KO) in 1% emulgen 911 and 1 or 10 μM warfarin at 25° in a total volume of 0.25 mL. Warfarin as added prior to DTT addition, and KO was added 1 min after DTT addition. After 5 more min, the assays were quenched with 0.5 mL of 25 mM $HgCl_2$ and 1.5 mL of isopropanol:hexane (3:2, v/v). The hexane layer was removed, dried under nitrogen, and dissolved in 0.2 mL methanol. Vitamin K was quantitated via HPLC analysis as previously described [13]. Warfarin and bromadiolone inhibition curves were determined similarly except that both 4-hydroxycoumarins were dissolved in dimethyl sulfoxide rather than water, and pools of solubilized liver microsomes were used rather than individual livers. Apparent K_m values for the epoxide reductase substrates were calculated using microsomes from a pool of 12 normal mice or 8 bromadiolone-resistant mice. Vitamin K formation was linear over the 5-min assay period, and the initial rate data were fitted to the hyperbola $v = VA/(K + A)$ using Cleland's program HYPERO [23].

Chemicals. Sodium warfarin was a gift from the Wisconsin Alumni Research Foundation (Madison, WI), while bromadiolone was a gift from LiphaTech Inc. (Milwaukee, WI). Vitamin K (phylloquinone) and TAPS were purchased from Sigma (St. Louis, MO), while sodium cholate was from Kodak (Rochester, NY) and DTT from Boehringer Mannheim (Indianapolis, IN). Emulgen 911 was from Kao Atlas (Tokyo, Japan) and HPLC grade solvents were from American Burdick & Jackson (Muskegon, MI). Vitamin K was converted to KO by the method of Tishler *et al.* [24], and the epoxide was purified as previously described [13].

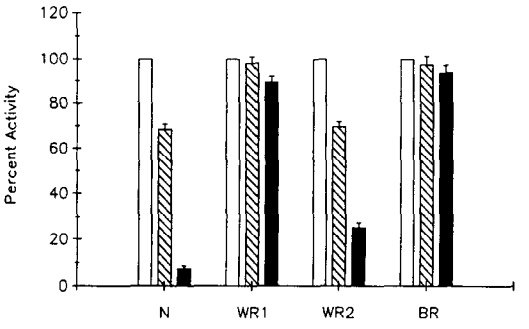

Fig. 1. Warfarin inhibition of the vitamin K epoxide reductase from normal (N), warfarin-resistant type 1 (WR1), warfarin-resistant type 2 (WR2), and bromadiolone-resistant (BR) mouse liver microsomes. Epoxide reductase activity in the presence of 1 μM (▨) or 10 μM (■) warfarin is expressed as the percentage of activity in the absence of warfarin (□). Values are the means ± SE for 27 N, 12 WR1, 7 WR2, and 20 BR mice. Epoxide reductase activities in the absence of warfarin (± SEM) were 0.114 ± 0.007, 0.036 ± 0.004, 0.107 ± 0.004, and 0.041 ± 0.003 nmol K/min/g liver for the N, WR1, WR2, and BR mouse liver microsomes.

RESULTS

When the warfarin sensitivity of the vitamin K epoxide reductase from normal (N), warfarin-resistant (WR), and bromadiolone-resistant (BR) Danish mice was examined, a wide range of warfarin sensitivity was apparent. Based on their warfarin inhibition profiles the warfarin-resistant mice could be divided easily into two different groups. They were designated warfarin-resistant type 1 (WR1: 10 μM warfarin inhibited <30% of the liver epoxide reductase activity) and warfarin-resistant type 2 (WR2: 10 μM warfarin inhibited >70% of the liver epoxide reductase activity. Although the division of animals into these two groups was based on an arbitrary selection of warfarin sensitivities, the relatively low coefficient of variance of both the warfarin sensitivity and the uninhibited reductase activity of the two groups strongly suggests that they represent different populations. The epoxide reductases from the BR and WR1 mice were very insensitive to warfarin inhibition (Fig. 1). The WR2 epoxide reductase appeared to be slightly less sensitive to warfarin inhibition than normal, but not nearly as insensitive as the BR and WR1 epoxide reductases. No difference was observed between the epoxide reductases from the male and female mice with respect to their sensitivity to coumarin inhibition or levels of activity (data not shown). The activities of the uninhibited vitamin K epoxide reductase in

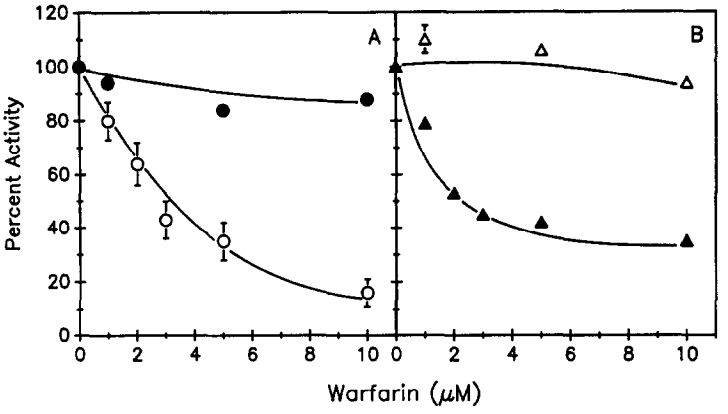

Fig. 2. Warfarin inhibition curves for the vitamin K epoxide reductase from normal (N) (○) and bromadiolone-resistant (BR) (●) mice (A), and warfarin-resistant type 1 (WR1) (△) and warfarin-resistant type 2 (WR2) (▲) mice (B). Each point and vertical bar represent the mean ± SEM for three experiments (N, BR, and WR1) or mean ± range for duplicates in one experiment (WR2), expressed as the percentage of epoxide reductase activity in the absence of warfarin. Activity levels in the absence of warfarin (± SEM) were 0.074 ± 0.004, 0.034 ± 0.003, 0.024 ± 0.004, and 0.065 ± 0.005 nmol K/min/g liver for N, BR, WR1, and WR2 liver microsomes, respectively.

liver microsomes of the different groups of mice are indicated in the legend to Fig. 1. It is apparent that the hepatic epoxide reductase activities of the normal and WR2 mice were similar. The epoxide reductase activities of the BR and WR1 mice were also similar, but these activities were only about 35% that of the activities of the enzyme measured in normal and WR2 mice.

The abilities of both warfarin and bromadiolone to inhibit the hepatic epoxide reductase in the four different mouse populations were also studied. The inhibition curves (Figs. 2 and 3) indicate that the hepatic epoxide reductase activities in the BR and WR1 mice were relatively insensitive to as much as 10 μM warfarin, but were inhibited about 50% by 10 μM bromadiolone. The warfarin-sensitive epoxide reductases from the N and WR-2 mice were inhibited 50% by 2–3 μM warfarin, and were even more sensitive to bromadiolone.

To obtain more information about the altered epoxide reductase observed in ther anticoagulant-resistant mice, kinetic parameters for the enzyme were measured in normal and BR mice (Table 1). The apparent K_m value for the reductant, DTT, was significantly lower in the BR strain. The V_{max} for epoxide reduction was also significantly lower in the

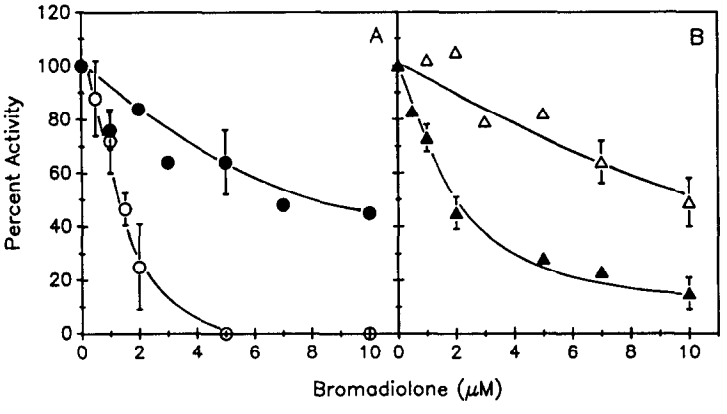

Fig. 3. Bromadiolone inhibition curves for the vitamin K epoxide reductase from normal (N) (○) and bromadiolone-resistant (BR) (●) mice (A), and warfarin-resistant type 1 (WR1) (△) and warfarin-resistant type 2 (WR2) (▲) mice (B). Each point and vertical bar represent the mean ± SEM for three experiments, expressed as a percentage of epoxide reductase activity in the absence of bromadiolone. Activity levels in the absence of bromadiolone (± SEM) were 0.064 ± 0.009, 0.032 ± 0.003, 0.030 ± 0.003, and 0.058 ± 0.006 nmol K/min/g liver for N, BR, WR1, and WR2 liver microsomes, respectively.

Table 1. Kinetic constants for the vitamin K epoxide reductase from normal and bromadiolone-resistant (BR) strain mice

Mice	K_m (app) KO (μM)	K_m (app) DTT (mM)	V_{max} (app) (nmol K/min/g liver)
Normal	28 ± 3	0.36 ± 0.03	0.230 ± 0.007
BR	30 ± 6	0.10 ± 0.02*	0.142 ± 0.008*

The apparent K_m for vitamin K epoxide (KO) was measured in the presence of 2 mM dithiothreitol (DTT), and KO concentrations varied from 6.25 to 200 μM. The apparent K_m for DTT measured in the presence of 40 μM KO, and DTT concentrations varied from 0.06 to 5.0 mM. Values are means ± SEM for three assays on microsomes pooled from 12 normal and 8 BR mice.

Significantly different (t-test) from the corresponding values of the normal enzyme at $P < 0.005$.

BR than in the N mouse population. Kinetic parameters for the epoxide reductase from the warfarin-resistant mice could not be determined due to an insufficient supply of these mice.

Warfarin sensitivity of the liver microsomal epoxide reductase was also assessed in wild mice trapped on warfarin-baited farms or unbaited farms in rural Colorado. Warfarin sensitivity of the enzyme was determined at a concentration of 10 μM warfarin (Fig. 4). Only one of 21 mice obtained from unbaited farms had a hepatic epoxide reductase that retained greater than 30% of uninhibited activity in the presence of 10 μM warfarin, while 4 of 18 mice trapped on baited farms showed this degree of warfarin insensitivity. The distribution of warfarin insensitivity was not statistically significant (chi square test). The epoxide reductase activity in the absence of warfarin as 0.21 ± 0.02 nmol K/min/g liver for the mice from the unbaited farms and 0.066 ± 0.008 nmol K/min/g liver for the mice from the baited farms.

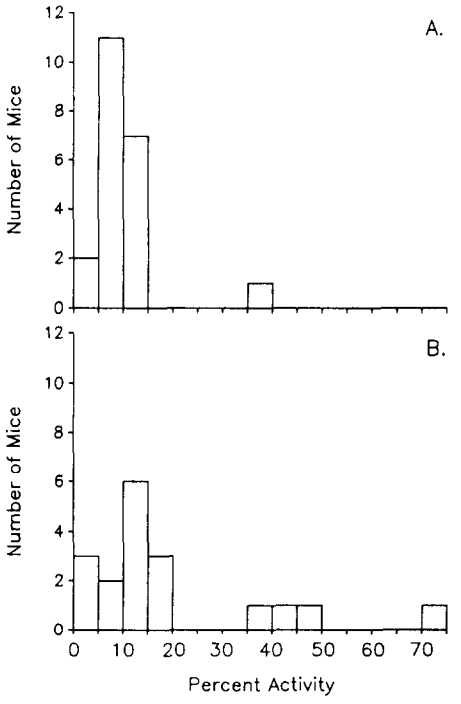

Fig. 4. Distribution histograms of the *in vitro* warfarin sensitivity of the vitamin K epoxide reductase from mice trapped on unbaited (A) or rodenticide-baited (B) farms. Percent activity in the presence of 10 μM warfarin relative to controls (no warfarin) was measured. The average control levels of activity (± SEM) were 0.21 ± 0.02 and 0.066 ± 0.008 nmol K/min/g liver for the mice from unbaited and baited farms, respectively.

DISCUSSION

These data indicate that the mechanism of warfarin resistance in some Danish genetically resistant populations of mice (BR and WR1) is analogous to that observed in some rats. The bromadiolone-resistant and the most resistant (WR1) of the two warfarin-resistant phenotypes had a hepatic vitamin K epoxide reductase that was relatively insensitive to *in vitro* inhibition by warfarin and bromadiolone. This is analogous to the biochemical basis of resistance in a Welsh strain of warfarin-resistant rats. The epoxide reductase activity in these resistant mice was even less sensitive to warfarin inhibition than has been demonstrated previously for the Welsh rat strain [9, 10, 25]. The altered rat epoxide reductase can be inhibited *in vitro* by 10–50 μM warfarin, whereas the epoxide reductases in these mouse strains were inhibited by only 20% with 100 μM warfarin (data not shown). The K_m of the epoxide reductase for DTT was lower in the Welsh-resistant rat strain than in warfarin-sensitive rats, whereas the K_m of the enzyme for KO was similar in both rat strains [26]. An analogous situation was observed with the normal and bromadiolone-resistant mouse strains studied here. It is not known whether the bromadiolone-resistant mice have an elevated vitamin K requirement as had been observed for the Welsh strain warfarin-resistant rats [27, 28], but other than that observation, the mechanism of warfarin resistance in these mice appears to be the same as that observed in the Welsh strain rats. This is consistent with the analogous linkage position of the gene for warfarin resistance in mice and rats. The available data also suggest that the BR and WR1 resistant mouse populations may, in fact, be the same. It is likely that the WR1 warfarin-resistant mouse strains would have also been resistant to a bromadiolone test diet, but they were not subjected to this protocol.

The mechanism of warfarin resistance in the second mouse strain (WR2) is not as obvious. These mice have an epoxide reductase that is only slightly less sensitive to warfarin inhibition than normal. This difference alone is unlikely to be sufficient to explain the warfarin resistance of the population, and it is possible that the mechanism of resistance in this population is similar to that previously described in England [18]. Complete [12, 29] or partial reversibility [13] of the warfarin inhibition,

and a rapid clearance of warfarin [13] have been shown to be factors in warfarin resistance in rats, and may be involved in this mouse strain. Sutcliffe *et al.* [30] have reported that a population of warfarin-resistant mice had a 2.5-fold higher overall rate of warfarin metabolism in their liver microsomes, and have demonstrated that an alteration in cytochrome P450 isozymes was responsible for this difference. These studies have not included measurements of epoxide reductase sensitivity, so the importance of alterations in warfarin metabolism in the resistance is not known.

The data in Fig. 4 indicate that warfarin sensitivity of mice trapped in Colorado also varied considerably. Only 1 of 21 mice trapped in an area not known to have been baited with anticoagulant rodenticides had a hepatic epoxide reductase that retained over 30% activity in the presence of 10 μM warfarin, whereas 4 of 18 mice trapped from baited farms had an enzyme with this degree of warfarin insensitivity. The enzyme activity was much lower in mice from the baited area farms, which would be consistent with warfarin resistance. However, the effects of rodenticide ingestion on epoxide reductase activity do persist for an extended time [31], and this may be the cause of the decreased activity.

Acknowledgements—This research was supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison, and in part by Grants GM-07215, DK-14881 and HL-29586 from the National Institutes of Health, Grant BSR 8909172 from the National Science Foundation, and a Grant from the National Pest Control Association.

REFERENCES

1. Boyle CM, Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisons. *Nature* **188**: 517, 1960.
2. Lund M, Resistance to the second-generation anticoagulant rodenticides. *Proc Vertebr Pest Conf* **11**: 89–94, 1984.
3. Jackson WB, Ashton AD and Delventhal K, Overview of anticoagulant rodenticide usage and resistance. In: *Current Advances in Vitamin K Research* (Ed. Suttie JW), pp. 381–388. Elsevier Science Publishers, New York, 1988.
4. Bishop JA. A neoDarwinian approach to resistance: Examples from mammals. In: *Genetic Consequences of Man Made Change* (Eds. Bishop JA and Cooks LM), pp. 37–57. Academic Press, New York, 1981.
5. Dodsworth E, Mice are spreading despite such poisons as warfarin. *Municipal Engng* **3746**: 1668, 1961.
6. Morgan DG and Petras ML, Variability in response to warfarin in natural populations of the house mouse. *Genetics* **91**: S84, 1979.
7. Siddiqi Z and Blaine WD, Anticoagulant resistance in house mice in Toronto, Canada. *Environ Health Rev* **26**: 49–51, 1982.
8. Zimmerman A and Matschiner JT, Biochemical basis of hereditary resistance to warfarin in the rat. *Biochem Pharmacol* **23**: 1033–1040, 1974.
9. Hildebrandt EF and Suttie JW. Mechanism of coumarin action: Sensitivity of vitamin K metabolizing enzymes of normal and warfarin-resistant rat liver. *Biochemistry* **21**: 2406–2411, 1982.
10. Hazelett SE and Preusch PC, Tissue distribution and warfarin sensitivity of vitamin K epoxide reductase. *Biochem Pharmacol* **37**: 929–934, 1988.
11. MacNicoll AD, A comparison of warfarin resistance and liver microsomal vitamin K epoxide reductase activity in rats. *Biochim Biophys Acta* **840**, 13–20, 1985.
12. Thijssen HHW, Warfarin resistance: Vitamin K epoxide reductase of Scottish resistance genes is not irreversibly blocked by warfarin. *Biochem Pharmacol* **36**: 2753–2757, 1987.
13. Misenheimer TM and Suttie JW, Warfarin resistance in a Chicago Strain of rats. *Biochem Pharmacol* **40**: 2079–2084, 1990.
14. Pool JG, O'Reilly RA, Schneiderman LJ and Alexander M, Warfarin resistance in the rat. *Am J Physiol* **215**: 627–631, 1968.
15. Greaves JH and Ayres P, Inheritance of Scottish-type resistance to warfarin in the Norway rat. *Genet Res* **26**: 231–239, 1976.
16. Greaves JH and Ayres PB, Multiple allelism at the locus controlling warfarin resistance in the Norway rat. *Genet Res.* **40**: 59–64, 1982.
17. Wallace ME and MacSwinney FJ, A major gene controlling warfarin-resistance in the house mouse. *J Hyg* **76**: 173–181, 1976.
18. MacNicoll AD and Dean RH,Warfarin inhibition of hepatic vitamin K epoxide reductase activity in warfarin-susceptible and -resistant house mice (*Mus domesticus*). *Pestic Biochem Physiol* **44**: 68–73, 1992.
19. Lund M, The "second generation" anticoagulants: A review. *Acta Zool Fenn* **173**: 149–153, 1985.
20. Rowe FP and Redfern R, Toxicity tests on suspected warfarin-resistant house mice (*Mus musculus* L.). *J Hyg* **63**: 417–425, 1965.
21. Redfern R and Gill JE, Laboratory evaluation of bromadiolone as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. *J Hyg* **84**: 263–268, 1980.
22. Salmon TP and Marsh RE. Age as a factor in rodent susceptibilty to rodenticides–A review. In: *Vertebrate Pest Control and Management Materials* (Ed. Beck JK), pp. 84–98. American Society for Testing and Materials, Philadelphia, 1979.
23. Cleland WW, Determining the chemical mechanism of enzyme-catalyzed reactions by kinetic studies. *Adv Enzymol* **45**: 273–387, 1977.
24. Tishler M, Fieser LF and Wendler NL, Hydro, oxido, and other derivatives of vitamin K_1 and related compounds. *J Am Chem Soc* **62**: 2866–2871, 1940.
25. Fasco MJ, Hildebrandt EF and Suttie JW, Evidence that warfarin anticoagulant action involves two distinct reductase activities. *J Biol Chem* **257**: 11210–11212, 1982.
26. Hildebrandt EF, Preusch PC, Patterson JL and Suttie JW. Solubilization and characterization of vitamin K epoxide reductase from normal and warfarin-resistant rat liver microsomes. *Arch Biochem Biophys* **228**: 480–492, 1984.
27. Henderson MA, Suttie JW and Link KP, Warfarin metabolism and vitamin K requirement in the warfarin-resistant rat. *Am J Physiol* **217**: 1316–1319, 1969.
28. Greaves JH and Ayres P, Warfarin resistance and vitamin K requirement in the rat. *Lab Anim* **7**: 141–148, 1973.
29. Vermeer C, Soute BAM, Aalten M, Knapen MHJ and Thijssen HHW, Vitamin K reductases in normal and warfarin-resistant rats. *Biochem Pharmacol* **37**: 2876–2878, 1988.
30. Sutcliffe FA, MacNicoll AD and Gibson GG, Hepatic microsomal warfarin metabolism in warfarin-resistant

and susceptible mouse strains: Influence of pretreatment with cytochrome P-450 inducers. *Chem Biol Interact* **75**: 171–184, 1990.
31. Mosterd JJ and Thijssen HHW, The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Br J Pharmacol* **104**: 531–535, 1991.