Elsevier Editorial System(tm) for Food and Chemical Toxicology

Manuscript Draft

Manuscript Number: FCT-D-17-01206R3

Title: 7-hydroxylation of warfarin is strongly inhibited by sesamin, but not by episesamin, caffeic and ferulic acids in human hepatic microsomes

Article Type: Short Communication

Keywords: S-7-hydroxywarfarin; cytochrome P450; in vitro; food-drug interactions; detoxification

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Abstract: Warfarin is a commonly used anticoagulant drug and is a derivate of coumarin. Cytochrome P450 2C9 (CYP2C9) plays the key role in transformation of coumarin and thus, influences determination of warfarin dosage. A number of factors including dietary compounds such as sesamin, caffeic acid and ferulic acids can regulate the activity of CYP2C9. The present study tested the hypothesis that sesamin, episesamin, caffeic acid and ferulic acid decreases the rate of warfarin 7-hydroxylation via inhibition of hepatic CYP2C9. The experiments were conducted on hepatic microsomes from human donors. It was demonstrated that the rate of 7-hydroxylation of warfarin was significantly decreased in the presence of sesamin in the range of concentrations from 5 to 500 nM, and was not affected by episesamin, caffeic acid and ferulic acid in the same range of concentrations. The kinetic analysis indicated non-competitive type of inhibition by sesamin with Ki = 202±18 nM. In conclusion, the results of our in vitro study revealed that sesamin was able to inhibit formation of a major metabolite of warfarin, 7-hydroxywarfarin. The potentially negative consequences of the consumption of high amounts of sesamin-containing food or dietary supplements in warfarin-treated patients need to be further studied.

Response to Reviewers: Dear Editor,

The authors greatly appreciates the time and valuable comment given by the editor and reviewers in order to improve our manuscript. We provide a detailed answer to every comment below.

Reviewer #1: I think the authors revised the paper appropriately according to the comments.
However, I wonder whether there is a specificity of sesamin on CYP2C9, because they did not check the effect on other CYPs. In figure 2, inhibitory effect was only detected in "no preincubation condition". If sesamin directly denature proteins including CYP enzymes, same phenomenon would be detected in other CYPs, such as CYP3A4.

Response:
In the present study we did not measure activities of specific isoforms, but focused on warfarin 7-hydroxylation since it is a major pathway of warfarin metabolism. It is known that 7-hydroxywarfarin is metabolised by CYP2C9 (Yasuda et al., 2010); thus we suggest that inhibition of warfarin 7-hydroxylation in the presence of sesamin is due to competitive inhibition of CYP2C9. Inhibitory effect was only detected if no preincubation step was included, indicating that the nature of the inhibition is reversible, so no direct denaturation of proteins occurred.

Minor comments
In highlight No.2, please include episesamin in addition to caffeic acid and ferulic acid.
Response:This comment was addressed; highlight 2 were updated according to the suggestion

In page 3 line 2, please mention the relationship between episesamin and sesamin as described in page 5 line 4.
I think it is better to mention that same amount of seamin and episesamin are found in sesame oil and dietary supplement.
Response: This comment was addressed. It was also mentioned that episesamin is a geometric isomer of sesamin

Reviewer #2: The authors addressed my comments only partly. They simply inserted the points raised in my previous assessment into revised text. However, these issues (stereospecificity of interactions between warfarines and PXR, and CYPs, and PXR antagonism of sesamine)are not integrated in meaningfull formate throughout the paper. The authors should comprise broadly these issues including the integration with the achievements of their current study.

Response: Thank you for this comment. We agree that it would be very interesting to investigate the role of nuclear receptors. However this was not the aim of our study and it would not be possible to conduct this research on the in vitro model which we used in this study (microsomes). A reason for this is that microsomes contain CYP450, but not receptors or transporters. Microsomes as a model are commonly used to study metabolism of selected compounds and/or metabolism inhibition. We have added further comments related to this aspect to the Discussion section. We would like to highlight that results of our study are novel and provide, for the first time, an important information on possible interactions between sesamin and warfarin hepatic metabolism
Investigation of the role of nuclear receptors and possibly other factors in the inhibition of warfarin metabolism by sesamin will be an aim of our future studies on another model which would be suitable for answering these questions (e.g. isolated hepatocytes).

Cover Letter

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Institution för Molekulära vetenskaper
Department of Molecular Sciences

26 December, 2017

Dear Editor,

Thank you for evaluation of our manuscript. We would like to submit the revised version of the manuscript "***7-hydroxylation of warfarin is strongly inhibited by sesamin, but not by caffeic and ferulic acids in human hepatic microsomes***" by Pilipenko et al.

The revision was performed according to the suggestions from Editor and reviewers responses. We have done our outmost to deal with every comment raised, and provide a detailed answer to every comment.

Thank you for your consideration. We look forward to hearing from you.

Sincerely yours,

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7-hydroxylation of warfarin is strongly inhibited by sesamin, but not by episesamin, caffeic and ferulic acids in human hepatic microsomes

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Abstract

Warfarin is a commonly used anticoagulant drug and is a derivate of coumarin. Cytochrome P450 2C9 (CYP2C9) plays the key role in transformation of coumarin and thus, influences determination of warfarin dosage. A number of factors including dietary compounds such as sesamin, caffeic acid and ferulic acids can regulate the activity of CYP2C9. The present study tested the hypothesis that sesamin, episesamin, caffeic acid and ferulic acid decreases the rate of warfarin 7-hydroxylation via inhibition of hepatic CYP2C9. The experiments were conducted on hepatic microsomes from human donors. It was demonstrated that the rate of 7-hydroxylation of warfarin was significantly decreased in the presence of sesamin in the range of concentrations from 5 to 500 nM, and was not affected by episesamin, caffeic acid and ferulic acid in the same range of concentrations. The kinetic analysis indicated non-competitive type of inhibition by sesamin with Ki = 202±18 nM. In conclusion, the results of our *in vitro* study revealed that sesamin was able to inhibit formation of a major metabolite of warfarin, 7-hydroxywarfarin. The potentially negative consequences of the consumption of high amounts of sesamin-containing food or dietary supplements in warfarin-treated patients need to be further studied.

Keywords: S-7-hydroxywarfarin; cytochrome P450; in vitro; food-drug interactions; detoxification

1. Introduction

Warfarin, a derivative of coumarin, is an oral anticoagulant drug to manage thromboembolic disease by preventing the synthesis of vitamin K-dependent proteins involved in blood coagulation, predominantly factors II, VII, IX and X (Rice et al., 2003). It is clinically used as a racemic mixture of equal amounts of S and R stereoisomers, with *S*-warfarin having 3-5 times greater anticoagulation potency compared to R-warfarin. The predominant fate of *S*-warfarin biotransformation in humans is 7-hydroxylation of the coumarin ring by cytochrome P450 (CYP) 2C9 (Yasuda et al., 2010). This metabolic transformation of warfarin to 7-hydroxywarfarin by CYP2C9 is specific only for S-warfarin. Therefore, polymorphisms in CYP2C9 have effect on the rate of coumarin biotransformation and thus are the major determinants of warfarin dosage requirement (Cavallari et al., 2011). Because of a narrow therapeutic index of warfarin and its predisposition to drug interactions, careful studies are required to identify foods or food components with potential to interact with warfarin metabolism, and explain biochemical mechanisms causing these interactions (Leite et al., 2016). Nowadays, at least 58 plants including ginger, garlic and St. John's wort are known to interact with warfarin metabolism (Leite et al., 2016). The majority of such interactions are due to inhibition of expression or activity of CYP2C9 or other enzymes involved in warfarin metabolism (Di Minno et al., 2017). Additionally, clinical factors, such as weight, age, smoking, concomitant use of other drugs that interfere with warfarin metabolism, are known to affect warfarin dose requirements and cause the difficulties of anticoagulant management (Gage et al., 2004). The effect of vitamin K intake on warfarin anticoagulation is a classic example of warfarin-food interactions. Therefore, it is of increasing interest to development of new vitamin K-independent coagulation factor inhibitors which lack food compound interactions and can replace warfarin. Yet, warfarin remains nowadays the most commonly prescribed oral anticoagulant.

Sesame *(Sesamun indicum L.)* is an important food source in many countries and is often associated with decreased risk of cardiovascular diseases (Namiki et al., 2007). It has also been suggested to have anti-oxidant and anti-inflammatory properties (Kamal-Eldin et al., 2011). These properties are mainly attributed to the lignans in sesame-based products sesamin, sesamol and sesamolin. The highest observed concentration (Cmax) of sesamin in human plasma was reported to be 100 nM after consumption of a single dose of sesame seeds (50 g) (Peñalvo et al., 2005), and was 8 nM after intake of 50 mg of sesamin supplement containing an equal amount of sesamin and episesamin (Tomimori et al., 2013). The maximum concentration of sesamin in the human liver following a 50 mg oral administration was estimated to be 130 nM (Yasuda et al., 2015).

Similarly to warfarin, sesamin is metabolized by CYP2C9 and also act as a mechanism-based inhibitor of CYP2C9 activity in human liver microsomes (Yasuda et al., 2010). Although sesamin was demonstrated not to interfere with warfarin metabolism in rats (Yasuda et al., 2015), the safety of sesamin with respect to warfarin–sesamin interactions in humans are not yet clarified.

Caffeic acid, the major representative of hydroxycinnamic acids, is one of the most common phenolic acids found in coffee, fruits and dietary supplements. Caffeic acid was found to be a potent competitive inhibitor of CYP2C9 (Rastogi et al., 2014), the enzyme which mediates warfarin metabolism. Ferulic acid, which is a metabolite of caffeic acid, is commonly found in rice, wheat, oats, grasses, flowers, fruits and nuts, and was also recently shown to interact with warfarin (Li et al., 2016).

Although warfarin has been widely investigated, particularly in its relationship to vitamin K intake, the effects of commonly occurring food compounds on warfarin metabolism remain largely uncharacterized. It is known that the efficacy of warfarin is mainly affected when metabolism of S-warfarin is altered. Thus, in the present study we tested the hypothesis that

sesamin, caffeic and ferulic acid decrease the rate of warfarin 7-hydroxylation through inhibition of human hepatic CYP2C9 *in vitro*. Effect of episesamin on warfarin metabolism was also investigated, as the same amount of both, sesamin and its geometric isomer episesamin is present in sesame oil and dietary supplement.

2. Materials and methods

2.1. Materials and reagents

Warfarin, 7-hydroxywarfarin, episesamin, caffeic acid, ferulic acid, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), dimethylsulfoxide (DMSO) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Steinheim, Germany). Sesamin was obtained from Kebiotech (Beijing, China). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). A stock solutions (20 mM) of sesamin, episesamin, caffeic and ferulic acids were prepared in dimethyl sulfoxide (DMSO). Human liver microsomes pooled from individual donors of both genders were purchased from three companies, GIBCO (Stockholm, Sweden, Cat.HMMCPL, lot. PL050B-B), inVitro (Stockholm, Sweden, Product X008070, lot SPL), and Sigma-Aldrich (Steinheim, Germany, pooled lots SLBM1949V and SLBK2748V). The pool from GIBCO contained microsomes from 20 donors and the pool from inVitro - from 150 donors. The number of donors in the pool obtained from Sigma-Aldrich was not specified. The samples from three purchasing source were selected because we have previously observed between-manufacture differences in the activity of selected CYPs (unpublished data). The protein concentration in all human liver microsomes was 20 mg/mL.

2.2. Inhibition study

The rate of warfarin 7-hydroxylation was determined as described previously (Yamaori et al., 2015). Concentrations of 7-hydroxywarfarin were determined by interpolation of standard curve produced with authentic standards.

Sesamin, episesamin, caffeic and ferulic acid were added into the incubation mixture at final concentrations of 5, 10, 50, 100, and 500 nM. The amount of 7-hydroxywarfarin formed in the presence of sesamin, episesamin, caffeic or ferulic acid was compared with control incubations containing corresponding amounts of DMSO. All inhibitors were added before the addition of warfarin. To test reversibility of the inhibition, the test inhibitors were first pre-incubated with human liver microsomes for 30 min at 37 °C with NADPH but without warfarin. After this, warfarin was added and the mixture was incubated for further 60 min. The two control incubations (one without a pre-incubation step and another with pre-incubation in absence of NADPH) were run in parallel. An experiment with a known competitive inhibitor of CYP2C9 sulfaphenazole was conducted as a positive control using 0.1, 0.5, 12.5 and 50 µM of sulfaphenazole without pre-incubation step (Bourrié et al., 1996).

2.3. Data analysis

The IC50 values (concentration causing 50% reduction of control activity) for sesamin were determined by linear regression analysis from plotting the logarithm of inhibitor concentration vs percentage of activity remaining after inhibition (GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego California, USA). Inhibition constant (Ki) was determined using range of sesamin (10 and 50 nM) and warfarin (5 to 200 µM) concentrations. Michaelis-Menten constant (Km) and maximum velocity (Vmax) were calculated using a non-linear regression analysis. Enzyme kinetic equations for competitive, non-competitive, uncompetitive and mixed inhibition type were compared using extra least sum-of-squares F test.

3. Results and discussion

As expected, sulfaphenazole inhibited CYP2C9-mediated warfarin 7-hydroxylation in a concentration-dependent manner (Fig. 1). Sesamin inhibited warfarin 7-hydroxylation in all the tested concentrations, while its geometric isomer episesamin had no effect (Fig. 2). The lowest amount of 7- hydroxywarfarin was formed in the presence of 500 nM of sesamin (mean ± standard deviation; 46 ± 8.5 %). Including a pre-incubation step did not increase sesamin inhibitory effect as indicated by unaltered IC50 value (IC50 = 202 nM and 193 nM in the absence and presence of pre-incubation step, respectively). More detailed kinetic analysis indicated non-competitive type of inhibition (Fig. 3), with Ki = 202 ± 18 nM, a decrease in Vmax from 66.7 ± 8.9 to 48.6 ± 5.0 pmol/min/mg and unaltered Km = 7.2 ± 2.1 μM. Since warfarin has a narrow therapeutic index, its inhibition might have serious and difficult to predict consequences.

It is well documented that warfarin pharmacotherapy can be affected by food or dietary supplements in several ways (Nutescu et al., 2006). One example is inhibition of the anticoagulant effect of warfarin by the dietary vitamin K. Since warfarin function is to reduce the activity of vitamin K in blood clotting processes, dietary vitamin K might diminish the effect of warfarin (Nutescu et al., 2004; 2006). However, recent reports provided conflicting results (Violi et al., 2016; Choi et al., 2014; Kwon et al., 2010). It was suggested that stable dietary intake of vitamin K is more relevant for a reduction in variations of warfarin effect compared to previous recommendations to restrict vitamin K when taking warfarin (Violi et al., 2016).

Hepatic S-warfarin metabolism is mediated by CYP. Therefore, potent inhibitors or inducers of CYP activity might strongly modify the therapeutic effects of warfarin. The main finding of our study was that sesamin, the major fat-soluble lignan in sesame seed, inhibited

7-hydroxylation of warfarin with Ki = 202 nM. In the liver, sesamin is metabolized mainly by CYP2C9 to produce hydroxylated metabolites (Yasuda et al., 2010). The same enzyme is responsible for the formation of 7-hydroxywarfarin from warfarin. Thus, we expected that sesamin and warfarin will compete for the active site of the enzyme. Indeed, Yasuda et al. (2010) described a competitive inhibition of CYP2C9 by sesamin with the apparent Ki value of 24 μM. However, in the present study we observed non-competitive inhibition of warfarin metabolism, which means that sesamin binds to CYP2C9 in another place than the active site and alter the active site so that it can't bind warfarin. Previously it was suggested that sesamin can also act as a mechanism-based inhibition of CYP2C9 activity, estimated as diclofenac hydroxylation, with the apparent Ki value of 22 μM and K_{inact} value of 0.13 min^{-1}, respectively (Yasuda et al., 2010). It is obvious that non-competitive inhibition is less severe compared to the mechanism-based because it does not require re-synthesis of inactivated enzyme to restore its activity. However, in the present study, sesamin inhibited warfarin metabolism at much lower concentrations than in the study of Yasuda et al. (2010). The differences in inhibition mode and Ki values observed in the present study and previously published results are likely to be due to the use of different substrates. Yasuda et al. (2010; 2015) analysed diclofenac metabolism to estimate CYP2C9 activity, whilst the present study focuses on warfarin metabolism. Interestingly, no interactions between sesamin and diclofenac were observed when sesamin was administered to rats in a concentration of 100 mg/kg body weight (Yasuda et al., 2015), suggesting possible substrate- or species-related differences in the regulation of CYP2C9 activity by sesamin. The peak plasma concentration of sesamin in human was previously reported to be 100 nM after administration of a single dose of sesame seeds containing 170 mg of sesamin (Peñalvo et al., 2005), which is within the range of sesamin concentrations at which we observed inhibition of warfarin metabolism. Generally, the contents of sesamin in sesame-based food products is highly variable

(Moazzami et al., 2007). In refined sesame oils, sesamin concentrations ranged between 118 and 401 mg/100 g seed (Moazzami et al., 2007). Moreover, sesamin content greatly vary between the sesame cultivars depending on genetic variation and geographic location (Wang et al., 2013). Nowadays, consumption of products and cuisines with sesame seeds is steadily increasing, and sesamin supplements are considered by consumers as highly healthy. Thus, a clinically important interaction with concomitantly administrated warfarin and sesamin might be probable and need further investigations.

Episesamin, a geometric isomer of sesamin, did not affect warfarin metabolism in the present study. Differences in biological effects of sesamin and episesamin were observed previously in rats. It was found that episesamin increases gene expression and activity of the enzymes involved in fatty acid metabolism to a higher degree than sesamin (Kushiro et al., 2002; Ide et al., 2009). Episesamin differs from sesamin in the three-dimensional structure. Moreover, it was shown that episesamin is a poorer substrate of CYP2C9 than sesamin, and is also metabolised by CYP1A2 (Yasuda et al., 2012). Thus, the differences in metabolism of sesamin and episesamin might be responsible for the divergent effects of these compounds on warfarin metabolism.

In the present study, only direct inhibition of warfarin metabolism by several bioactive compounds was considered. However, warfarin metabolism can be altered through the activation/inhibition of pregnane X receptor (PXR). Warfarin itself and warfarin-hydroxylated racemic metabolites act as activators of PXR, and consequently induce PXR target genes including CYP3A4 and CYP2C9 (Rulcova et al., 2010; Miller, 2010). Interestingly, S-warfarin is a less potent PXR activator compared to R-warfarin (Rulcova et al., 2010). Sesamin was demonstrated to act as a PXR-antagonist, causing down-regulation of CYP3A4 (Lim et al., 2012). It is likely that sesamin also down-regulates *in vivo* CYP2C9 expression in a similar manner. In the present study, the role of nuclear receptors was not studied as they

not present in the model used (isolated microsomes). This aspect will be an aim of future study on other *in vitro* models which contain various nuclear receptors, conjugation enzymes and transporters. Our study provides important information on possibility of interactions between sesamin and warfarin hepatic metabolism. Thus, sesamin can affect warfarin metabolism by multiple mechanisms – directly, as demonstrated in the present study, and possibly thought the interaction with PXR. Moreover, CYP2C9-generated 7-hydroxywarfarin is also a potent inhibitor of CYP2C9 and limits metabolic capacity of CYP2C9 toward S-warfarin (Jones 2010). However, physiological significance of this feedback inhibition of CYP2C9 requires further investigations.

Neither caffeic nor ferulic acids affected *in vitro* warfarin metabolism in the range of concentrations from 5 to 500 nM (data not shown). These results agree with the study by Appiah-Opong et al. (2007) who found no effect of ferulic acid on CYP2C9 activity. Interaction between ferulic acid and pharmacokinetics of warfarin was recently demonstrated in a biliary drainage model with healthy rats, but these interactions were suggested to happen at the level of enterohepatic circulation of warfarin and were not due to metabolism (Li et al., 2016). Caffeic acid did not have any effect on warfarin metabolism in the present study, although it was previously suggested that caffeic acid inhibits CYP2C9 activity (Rastogi et al., 2014). It should be, however, emphasized that the previous *in vitro* study by Rastogi et al. (2014) used high concentrations of caffeic acid (from 0.14 to 100 μM), and the Ki value for CYP2C9 was determined in the presence of 1.25, 2.5, 5.0 and 10 μM of caffeic acid.

4. Conclusion

The results of our *in vitro* study revealed that sesamin was able to non-competitively inhibit formation of a major metabolite of warfarin, 7-hydroxywarfarin. This inhibition was characterized by low Ki value of 202 ± 18 nM. The potentially negative consequences of the

consumption of high amounts of sesamin-containing food or dietary supplements in warfarin-treated patients need to be further studied.

Acknowledgements

This study was financially supported by the Swedish University of Agricultural Sciences, NJ Faculty and by the University of the West of England. Support was given to N. Pilipenko by the EU-Programme Erasmus Mundus, Action 2 Partnership. The work by M.K. Rasmussen was supported by the Lundbeck Foundation. This work was also supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program).

Conflicts of interest statement

The authors declare no conflicts of interest.

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doi: 10.1124/dmd.112.045906

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Figure legends

Figure 1. Formation of 7-hydroxywarfarin (percentage of control activity) in the presence of CYP2C9 inhibitor sulfaphenazole in human liver microsomes. Control activity was determined in the absence of sulfaphenazole and was regarded as 100%. Dashed line represent 50% of control activity. Data represent mean values and standard error of mean (n = 3).

Figure 2. Formation of 7-hydroxywarfarin (percentage of control activity) in human liver microsomes in the presence of sesamin and episesamin. Control activity was determined in the absence of sesamin and was regarded as 100%. Dashed line represent 50% of control activity. Activities was measured with and without 30 min pre-incubation before the addition of warfarin. Data represent mean values and standard error of mean (n = 3).

Figure 3. Saturation kinetics for warfarin 7-hydroxylation by human liver microsomes in the absence and presence of sesamin. Data represent mean values and standard error of mean from three microsomal pools. Curves indicate model-generated values using the Michaelis-Menten equation. Insert: Lineweaver-Burk plot of warfarin metabolism inhibition (non-competitive) by sesamin in human liver microsomes. Data represent mean values and standard error of mean (n = 3).

1. Introduction

Warfarin, a derivative of coumarin, is an oral anticoagulant drug to manage thromboembolic disease by preventing the synthesis of vitamin K-dependent proteins involved in blood coagulation, predominantly factors II, VII, IX and X (Rice et al., 2003). It is clinically used as a racemic mixture of equal amounts of S and R stereoisomers, with *S*-warfarin having 3-5 times greater anticoagulation potency compared to R-warfarin. The predominant fate of *S*-warfarin biotransformation in humans is 7-hydroxylation of the coumarin ring by cytochrome P450 (CYP) 2C9 (Yasuda et al., 2010). This metabolic transformation of warfarin to 7-hydroxywarfarin by CYP2C9 is specific only for S-warfarin. Therefore, polymorphisms in CYP2C9 have effect on the rate of coumarin biotransformation and thus are the major determinants of warfarin dosage requirement (Cavallari et al., 2011). Because of a narrow therapeutic index of warfarin and its predisposition to drug interactions, careful studies are required to identify foods or food components with potential to interact with warfarin metabolism, and explain biochemical mechanisms causing these interactions (Leite et al., 2016). Nowadays, at least 58 plants including ginger, garlic and St. John's wort are known to interact with warfarin metabolism (Leite et al., 2016). The majority of such interactions are due to inhibition of expression or activity of CYP2C9 or other enzymes involved in warfarin metabolism (Di Minno et al., 2017). Additionally, clinical factors, such as weight, age, smoking, concomitant use of other drugs that interfere with warfarin metabolism, are known to affect warfarin dose requirements and cause the difficulties of anticoagulant management (Gage et al., 2004). The effect of vitamin K intake on warfarin anticoagulation is a classic example of warfarin-food interactions. Therefore, it is of increasing interest to development of new vitamin K-independent coagulation factor inhibitors which lack food compound interactions and can replace warfarin. Yet, warfarin remains nowadays the most commonly prescribed oral anticoagulant.

Sesame *(Sesamun indicum L.)* is an important food source in many countries and is often associated with decreased risk of cardiovascular diseases (Namiki et al., 2007). It has also been suggested to have anti-oxidant and anti-inflammatory properties (Kamal-Eldin et al., 2011). These properties are mainly attributed to the lignans in sesame-based products sesamin, sesamol and sesamolin. The highest observed concentration (Cmax) of sesamin in human plasma was reported to be 100 nM after consumption of a single dose of sesame seeds (50 g) (Peñalvo et al., 2005), and was 8 nM after intake of 50 mg of sesamin supplement containing an equal amount of sesamin and episesamin (Tomimori et al., 2013). The maximum concentration of sesamin in the human liver following a 50 mg oral administration was estimated to be 130 nM (Yasuda et al., 2015).

Similarly to warfarin, sesamin is metabolized by CYP2C9 and also act as a mechanism-based inhibitor of CYP2C9 activity in human liver microsomes (Yasuda et al., 2010). Although sesamin was demonstrated not to interfere with warfarin metabolism in rats (Yasuda et al., 2015), the safety of sesamin with respect to warfarin–sesamin interactions in humans are not yet clarified.

Caffeic acid, the major representative of hydroxycinnamic acids, is one of the most common phenolic acids found in coffee, fruits and dietary supplements. Caffeic acid was found to be a potent competitive inhibitor of CYP2C9 (Rastogi et al., 2014), the enzyme which mediates warfarin metabolism. Ferulic acid, which is a metabolite of caffeic acid, is commonly found in rice, wheat, oats, grasses, flowers, fruits and nuts, and was also recently shown to interact with warfarin (Li et al., 2016).

Although warfarin has been widely investigated, particularly in its relationship to vitamin K intake, the effects of commonly occurring food compounds on warfarin metabolism remain largely uncharacterized. It is known that the efficacy of warfarin is mainly affected when metabolism of S-warfarin is altered. Thus, in the present study we tested the hypothesis that

sesamin, caffeic and ferulic acid decrease the rate of warfarin 7-hydroxylation through inhibition of human hepatic CYP2C9 *in vitro*. Effect of episesamin on warfarin metabolism was also investigated, as the same amount of both, sesamin and its geometric isomer episesamin is present in sesame oil and dietary supplement.

2. Materials and methods

2.1. Materials and reagents

Warfarin, 7-hydroxywarfarin, episesamin, caffeic acid, ferulic acid, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), dimethylsulfoxide (DMSO) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Steinheim, Germany). Sesamin was obtained from Kebiotech (Beijing, China). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). A stock solutions (20 mM) of sesamin, episesamin, caffeic and ferulic acids were prepared in dimethyl sulfoxide (DMSO). Human liver microsomes pooled from individual donors of both genders were purchased from three companies, GIBCO (Stockholm, Sweden, Cat.HMMCPL, lot. PL050B-B), inVitro (Stockholm, Sweden, Product X008070, lot SPL), and Sigma-Aldrich (Steinheim, Germany, pooled lots SLBM1949V and SLBK2748V). The pool from GIBCO contained microsomes from 20 donors and the pool from inVitro - from 150 donors. The number of donors in the pool obtained from Sigma-Aldrich was not specified. The samples from three purchasing source were selected because we have previously observed between-manufacture differences in the activity of selected CYPs (unpublished data). The protein concentration in all human liver microsomes was 20 mg/mL.

2.2. Inhibition study

The rate of warfarin 7-hydroxylation was determined as described previously (Yamaori et al., 2015). Concentrations of 7-hydroxywarfarin were determined by interpolation of standard curve produced with authentic standards.

Sesamin, episesamin, caffeic and ferulic acid were added into the incubation mixture at final concentrations of 5, 10, 50, 100, and 500 nM. The amount of 7-hydroxywarfarin formed in the presence of sesamin, episesamin, caffeic or ferulic acid was compared with control incubations containing corresponding amounts of DMSO. All inhibitors were added before the addition of warfarin. To test reversibility of the inhibition, the test inhibitors were first pre-incubated with human liver microsomes for 30 min at 37 °C with NADPH but without warfarin. After this, warfarin was added and the mixture was incubated for further 60 min. The two control incubations (one without a pre-incubation step and another with pre-incubation in absence of NADPH) were run in parallel. An experiment with a known competitive inhibitor of CYP2C9 sulfaphenazole was conducted as a positive control using 0.1, 0.5, 12.5 and 50 µM of sulfaphenazole without pre-incubation step (Bourrié et al., 1996).

2.3. Data analysis

The IC50 values (concentration causing 50% reduction of control activity) for sesamin were determined by linear regression analysis from plotting the logarithm of inhibitor concentration vs percentage of activity remaining after inhibition (GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego California, USA). Inhibition constant (Ki) was determined using range of sesamin (10 and 50 nM) and warfarin (5 to 200 µM) concentrations. Michaelis-Menten constant (Km) and maximum velocity (Vmax) were calculated using a non-linear regression analysis. Enzyme kinetic equations for competitive, non-competitive, uncompetitive and mixed inhibition type were compared using extra least sum-of-squares F test.

3. Results and discussion

As expected, sulfaphenazole inhibited CYP2C9-mediated warfarin 7-hydroxylation in a concentration-dependent manner (Fig. 1). Sesamin inhibited warfarin 7-hydroxylation in all the tested concentrations, while its geometric isomer episesamin had no effect (Fig. 2). The lowest amount of 7- hydroxywarfarin was formed in the presence of 500 nM of sesamin (mean ± standard deviation; 46 ± 8.5 %). Including a pre-incubation step did not increase sesamin inhibitory effect as indicated by unaltered IC50 value (IC50 = 202 nM and 193 nM in the absence and presence of pre-incubation step, respectively). More detailed kinetic analysis indicated non-competitive type of inhibition (Fig. 3), with Ki = 202 ± 18 nM, a decrease in Vmax from 66.7 ± 8.9 to 48.6 ± 5.0 pmol/min/mg and unaltered Km = 7.2 ± 2.1 μM. Since warfarin has a narrow therapeutic index, its inhibition might have serious and difficult to predict consequences.

It is well documented that warfarin pharmacotherapy can be affected by food or dietary supplements in several ways (Nutescu et al., 2006). One example is inhibition of the anticoagulant effect of warfarin by the dietary vitamin K. Since warfarin function is to reduce the activity of vitamin K in blood clotting processes, dietary vitamin K might diminish the effect of warfarin (Nutescu et al., 2004; 2006). However, recent reports provided conflicting results (Violi et al., 2016; Choi et al., 2014; Kwon et al., 2010). It was suggested that stable dietary intake of vitamin K is more relevant for a reduction in variations of warfarin effect compared to previous recommendations to restrict vitamin K when taking warfarin (Violi et al., 2016).

Hepatic S-warfarin metabolism is mediated by CYP. Therefore, potent inhibitors or inducers of CYP activity might strongly modify the therapeutic effects of warfarin. The main finding of our study was that sesamin, the major fat-soluble lignan in sesame seed, inhibited

7-hydroxylation of warfarin with Ki = 202 nM. In the liver, sesamin is metabolized mainly by CYP2C9 to produce hydroxylated metabolites (Yasuda et al., 2010). The same enzyme is responsible for the formation of 7-hydroxywarfarin from warfarin. Thus, we expected that sesamin and warfarin will compete for the active site of the enzyme. Indeed, Yasuda et al. (2010) described a competitive inhibition of CYP2C9 by sesamin with the apparent Ki value of 24 μM. However, in the present study we observed non-competitive inhibition of warfarin metabolism, which means that sesamin binds to CYP2C9 in another place than the active site and alter the active site so that it can't bind warfarin. Previously it was suggested that sesamin can also act as a mechanism-based inhibition of CYP2C9 activity, estimated as diclofenac hydroxylation, with the apparent Ki value of 22 μM and K_{inact} value of 0.13 min^{-1}, respectively (Yasuda et al., 2010). It is obvious that non-competitive inhibition is less severe compared to the mechanism-based because it does not require re-synthesis of inactivated enzyme to restore its activity. However, in the present study, sesamin inhibited warfarin metabolism at much lower concentrations than in the study of Yasuda et al. (2010). The differences in inhibition mode and Ki values observed in the present study and previously published results are likely to be due to the use of different substrates. Yasuda et al. (2010; 2015) analysed diclofenac metabolism to estimate CYP2C9 activity, whilst the present study focuses on warfarin metabolism. Interestingly, no interactions between sesamin and diclofenac were observed when sesamin was administered to rats in a concentration of 100 mg/kg body weight (Yasuda et al., 2015), suggesting possible substrate- or species-related differences in the regulation of CYP2C9 activity by sesamin. The peak plasma concentration of sesamin in human was previously reported to be 100 nM after administration of a single dose of sesame seeds containing 170 mg of sesamin (Peñalvo et al., 2005), which is within the range of sesamin concentrations at which we observed inhibition of warfarin metabolism. Generally, the contents of sesamin in sesame-based food products is highly variable

(Moazzami et al., 2007). In refined sesame oils, sesamin concentrations ranged between 118 and 401 mg/100 g seed (Moazzami et al., 2007). Moreover, sesamin content greatly vary between the sesame cultivars depending on genetic variation and geographic location (Wang et al., 2013). Nowadays, consumption of products and cuisines with sesame seeds is steadily increasing, and sesamin supplements are considered by consumers as highly healthy. Thus, a clinically important interaction with concomitantly administrated warfarin and sesamin might be probable and need further investigations.

Episesamin, a geometric isomer of sesamin, did not affect warfarin metabolism in the present study. Differences in biological effects of sesamin and episesamin were observed previously in rats. It was found that episesamin increases gene expression and activity of the enzymes involved in fatty acid metabolism to a higher degree than sesamin (Kushiro et al., 2002; Ide et al., 2009). Episesamin differs from sesamin in the three-dimensional structure. Moreover, it was shown that episesamin is a poorer substrate of CYP2C9 than sesamin, and is also metabolised by CYP1A2 (Yasuda et al., 2012). Thus, the differences in metabolism of sesamin and episesamin might be responsible for the divergent effects of these compounds on warfarin metabolism.

In the present study, only direct inhibition of warfarin metabolism by several bioactive compounds was considered. However, warfarin metabolism can be altered through the activation/inhibition of pregnane X receptor (PXR). Warfarin itself and warfarin-hydroxylated racemic metabolites act as activators of PXR, and consequently induce PXR target genes including CYP3A4 and CYP2C9 (Rulcova et al., 2010; Miller, 2010). Interestingly, S-warfarin is a less potent PXR activator compared to R-warfarin (Rulcova et al., 2010). Sesamin was demonstrated to act as a PXR-antagonist, causing down-regulation of CYP3A4 (Lim et al., 2012). It is likely that sesamin also down-regulates *in vivo* CYP2C9 expression in a similar manner. In the present study, the role of nuclear receptors was not studied as they

not present in the model used (isolated microsomes). This aspect will be an aim of future study on other *in vitro* models which contain various nuclear receptors, conjugation enzymes and transporters. Our study provides important information on possibility of interactions between sesamin and warfarin hepatic metabolism. Thus, sesamin can affect warfarin metabolism by multiple mechanisms – directly, as demonstrated in the present study, and possibly thought the interaction with PXR. Moreover, CYP2C9-generated 7-hydroxywarfarin is also a potent inhibitor of CYP2C9 and limits metabolic capacity of CYP2C9 toward S-warfarin (Jones 2010). However, physiological significance of this feedback inhibition of CYP2C9 requires further investigations.

Neither caffeic nor ferulic acids affected *in vitro* warfarin metabolism in the range of concentrations from 5 to 500 nM (data not shown). These results agree with the study by Appiah-Opong et al. (2007) who found no effect of ferulic acid on CYP2C9 activity. Interaction between ferulic acid and pharmacokinetics of warfarin was recently demonstrated in a biliary drainage model with healthy rats, but these interactions were suggested to happen at the level of enterohepatic circulation of warfarin and were not due to metabolism (Li et al., 2016). Caffeic acid did not have any effect on warfarin metabolism in the present study, although it was previously suggested that caffeic acid inhibits CYP2C9 activity (Rastogi et al., 2014). It should be, however, emphasized that the previous *in vitro* study by Rastogi et al. (2014) used high concentrations of caffeic acid (from 0.14 to 100 μM), and the Ki value for CYP2C9 was determined in the presence of 1.25, 2.5, 5.0 and 10 μM of caffeic acid.

4. Conclusion

The results of our *in vitro* study revealed that sesamin was able to non-competitively inhibit formation of a major metabolite of warfarin, 7-hydroxywarfarin. This inhibition was characterized by low Ki value of 202 ± 18 nM. The potentially negative consequences of the

consumption of high amounts of sesamin-containing food or dietary supplements in warfarin-treated patients need to be further studied.

Acknowledgements

This study was financially supported by the Swedish University of Agricultural Sciences, NJ Faculty and by the University of the West of England. Support was given to N. Pilipenko by the EU-Programme Erasmus Mundus, Action 2 Partnership. The work by M.K. Rasmussen was supported by the Lundbeck Foundation. This work was also supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program).

Conflicts of interest statement

The authors declare no conflicts of interest.

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Figure 1

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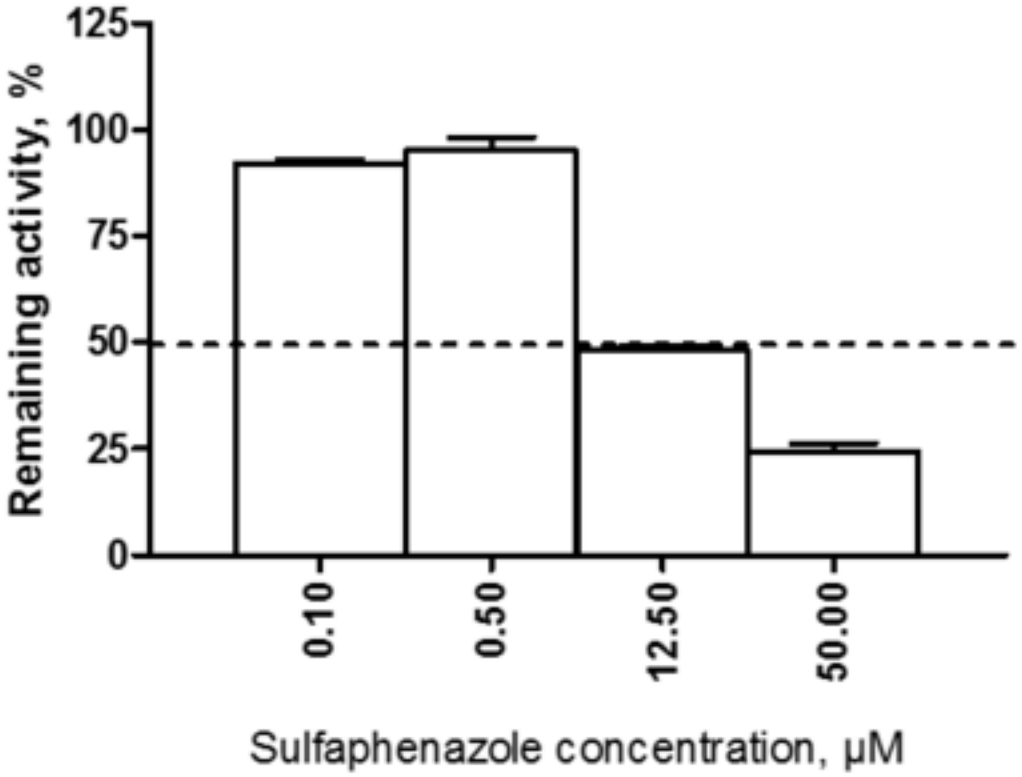

Figure 1

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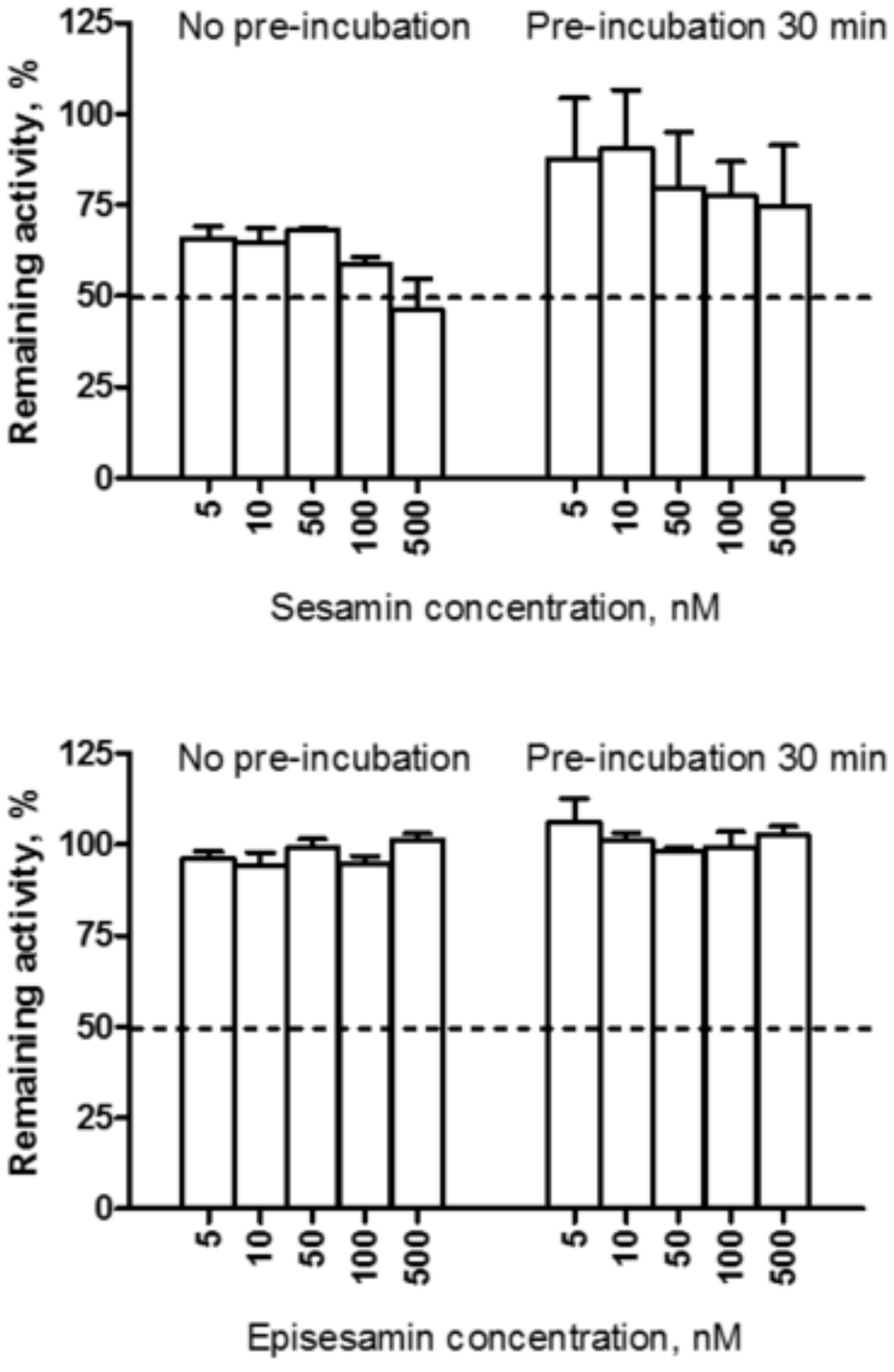

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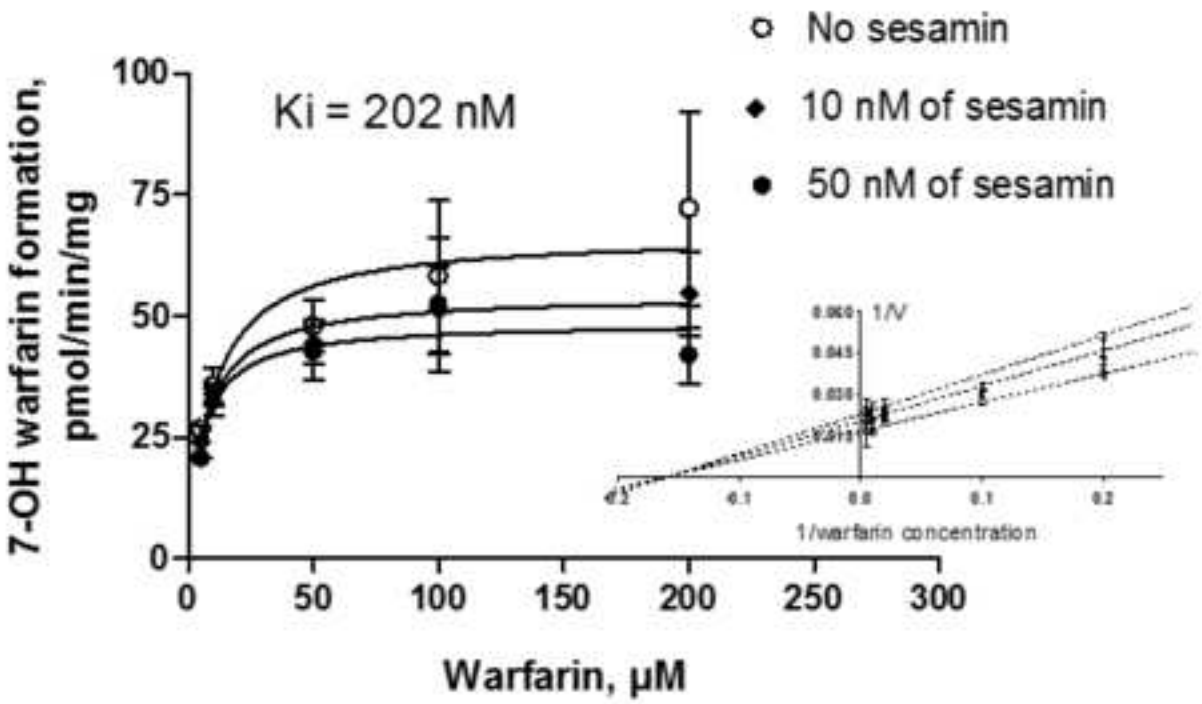

Figure 3

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