Development of a sandwich enzyme-linked immunosorbent assay (ELISA) to quantify γ-glutamyl-carboxylated clotting factor IX and assess redox susceptibility of anticoagulant chemicals

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ABSTRACT. Anticoagulant chemicals (ACCs) such as warfarin are widely used in medical applications as well as for their rodenticide properties. Their efficacy is greatly influenced by polymorphisms in the gene encoding vitamin K epoxide reductase (VKOR). Evaluation of the activity of ACCs toward VKOR variants is essential to determine their proper use. Presently, this is achieved by co-expressing VKOR of Rattus Norvegicus and human clotting factor IX in cultured cells and measuring inhibition of vitamin K-dependent gamma-glutamyl carboxylation of factor IX (glaFIX) activity. However, glaFIX has only been quantified using indirect methods like blood coagulation assays. We have developed a sandwich enzyme-linked immunosorbent assay using a glaFIX-specific antibody to quantify glaFIX and used this to analyze inhibition of VKOR activity by warfarin.

KEYWORDS: blood clotting factor, ELISA, vitamin-K-dependent protein, vitamin K epoxide reductase, warfarin

Anticoagulant chemicals (ACCs) such as warfarin have been used as rodenticides as well as in the medical field for over 70 years. These compounds inhibit the reduction of vitamin K epoxide (VKO) to vitamin K by vitamin K epoxide reductase (VKOR), resulting in decreased activity of vitamin-K-dependent clotting factors [12]. Clotting factors II, VII, IX, and X undergo vitamin-K-dependent conversion of a specific glutamic acid residue (Glu) to γ-carboxyglutamic acid (Gla) catalyzed by γ-glutamyl carboxylase (GGCX). Thus, ACC-mediated inhibition of VKOR leads to an excess of immature coagulation factor which is lacking the Glu to Gla conversion. An interesting pharmac/o/toxicological feature of ACCs is the resistance or hyper-susceptibility of VKOR to these compounds that is conferred by polymorphisms. There have been numerous reports of ACC-resistant rodents, many of which exhibit mutations in the vkorcl gene, which encodes dominant VKOR of mammalian hepatocyte (VKOR complex subunit 1) [6]. Highly toxic ACCs such as bromadiolone have been utilized to eliminate ACC-resistant rodents, but inadvertent poisoning of wild animals and pets has been reported [2, 7, 11]. In vitro studies of raptor VKORs have revealed that they were relatively more sensitive to warfarin compared to those of chicken and turkey [10]. On the contrary, the chicken and turkey VKORs were more tolerant to warfarin than Norwegian rat VKOR [17]. In addition, the large dose variability of warfarin among individuals is due, at least in part, to genetic polymorphisms of VKORC1 [1]. These findings highlight the importance of evaluating the influence that genetic polymorphisms and species differences have on the susceptibility of VKOR to ACC inhibition in order to ensure proper use of the compounds.

The in vivo redox partner (electron donor) of VKOR has not yet been identified. Therefore, current inhibition assays that evaluate the reduction of VKOR by ACCs typically involve in vitro measurement of VKOR activity using VKOR-localized liver microsomal fractions and dithiothreitol (DTT)—a common disulfide bond reductant—as the electron donor [5]. However, DTT directly reduces VKO, and the assay is not physiologically relevant. The results, therefore, may not provide an indication of the true sensitivity of VKOR. To address this, Fregin et al. developed a cultured-cell-based assay [4] which uses HEK293T cells transiently expressing VKOR variants and human clotting factor IX (FIX). The assay involves exposing the cells to VKO
and ACCs to evaluate inhibition of the production of γ-glutamyl-carboxylated FIX (glaFIX). The reduction of VKOR is induced by endogenous electron donors in cultured cells, thus creating a reactive environment that is similar to in vivo conditions. The reported 50% maximal inhibitory concentration (IC50) values correlate with in vivo warfarin sensitivity. There are two methods for measuring glaFIX in this cell-based assay; firstly, addition of FIX-secreted medium to FIX-deficient plasma followed by measurement of the clotting time using a coagulometer. The second method uses a chimeric protein of protein C and FIX. In this method, the expression of chimeric glaFIX-protein C— in which the Gla domain of protein C is replaced with the Gla domain of FIX—is quantified by enzyme-linked immunosorbent assay (ELISA). These assays do not enable direct quantification of glaFIX; rather, the level of γ-glutamyl carboxylation is used as a biomarker to evaluate ACC inhibition of activity. To address this problem, we have developed a method to directly quantify glaFIX involving a sandwich ELISA using an antibody specific for fully γ-glutamyl-carboxylated FIX [3].

The following optimized conditions were used for the sandwich ELISA: capture antibody—anti-glaFIX antibody (GMA-001; Green Mountain Antibodies, Burlington, VT, USA)—was diluted 500-fold in carbonate buffer (pH 9.6, 50 mM) and dispensed into a Maxisorp-treated 96-well microplate (Nunc™ 473709 Immuno™ Clear Standard Module Plate; Thermo Fisher Scientific, Waltham, MA, USA) at 100 µl/well. The plate was sealed and allowed to stand overnight at 4°C, then incubated at 37°C for 1 hr with shaking (150 rpm) to enhance coating. After washing once with tris-buffered saline supplemented with 0.05% Tween 20 (TBS-T), the plate was blocked using 200 µl of fish-gelatin blocking solution (Takara-Bio, Kusatsu, Japan). After a further three washes with TBS-T, 100 µl aliquots of specimens were added to each well and the plate incubated at 37°C for 2 min with shaking. Since the anti-glaFIX antibody requires calcium ions to bind to glaFIX, 10 mM CaCl2 (final concentration) was added to all wells. After washing five times with TBS-T supplemented with 5 mM CaCl2 (TBS-T-Ca), we added 100 µl/well of anti-FIX rabbit polyclonal antibody (GTX113689; GeneTex, Irvine, CA, USA) diluted 4,000-fold with Immuno Booster Solution 1 (Takara-Bio) and incubated the plate at 37°C for 1 hr with shaking. After washing five times with TBS-T-Ca, 100 µl/well of anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated preadsorbed (ab97080, Abcam PLC, Cambridge, UK) diluted 20,000-fold with TBS-T-Ca was added and the plate incubated for 30 min at room temperature with shaking. Next, 3,3′,5,5′-tetramethylbenzidine (TMB) soluble substrate solution (ELISA POD substrate POPULAR; Nacalai Tesque, Kyoto, Japan) was added at 100 µl/well and allowed to react for 3 min at room temperature in the dark. Then, 100 µl/well of 1 M sulfuric acid was added to terminate the reaction, and the absorbance at 450 nm measured immediately using a Multiskan FC (Thermo Fisher Scientific). All samples were measured in duplicate.

Figure 1 shows the calibration curves produced using human FIX protein standard (HCIX-0040; Hematalogic Technologies, Essex Junction, VT, USA) under the conditions described above. To examine the matrix effects of components in the medium, calibration curves were generated using samples diluted with antigen-antibody reaction-enhancing diluent (Signal Booster Neo, Beacle Inc.; Kyoto Japan) or cell assay medium (see below). Both curves showed good linear regression within the 0.525–420 ng/ml. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by the following equation.

LOD = 3.3 × s/a
LOQ = 10 × s/a

where s is the standard deviation of the absorbance of blanks, a is the intercept of the standard curve (n=4). The LOD of the medium was 0.18 ± 0.02 ng/ml, and that of the buffer was 0.23 ± 0.01 ng/ml. The LOQ of the medium was 0.55 ± 0.05 ng/ml, and

![Fig. 1. Standard curves of human γ-glutamyl carboxylation of factor IX (glaFIX) quantified by the sandwich enzyme-linked immunosorbent assay. Dilution of human FIX protein standards was carried out using reduced serum medium (Opti-MEM; 4% charcoal-stripped and heat-deactivated fetal bovine serum with 10 mM CaCl2) and antigen-antibody reaction-enhancing solution (Signal Booster Neo supplemented with 10 mM CaCl2). The x-axis shows the log10FIX concentration from 0.525–420 ng/ml. The y-axis represents the absorbance (with respective blanks subtracted). Data points depict mean ± standard error of the mean (n=4). There were no significant differences any of the regression parameters; slope, intercept, R2 value, LOD, and LOQ (multiple adjusted t-test, P<0.05). FIX, factor IX; FBS, fetal bovine serum; LOD, limit of detection; LOQ, limit of quantification.]
that of the buffer was 0.68 ± 0.03 ng/ml. To compare regression parameters (slope, intercept, R^2 value, LOD, and LOQ), multiple adjusted t-tests were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). There were no significant differences in any of the parameters (P<0.05).

The inclusion of calcium in the culture medium did not result in the formation of any precipitates, but white precipitate formed over time in the booster solution, even though it was a non-phosphate buffer. Also, TBS-T-Ca gradually produced calcium-derived precipitates after about 1 week of storage at room temperature. The reactivity of the TBS-T-Ca buffer as an antigen diluent also deteriorated (data not shown), possibly due to the stability of calcium ions in the solution. Since there was no cross-reactivity of bovine serum (FBS) that was added to the culture medium, culture medium was used as the diluent for calibration curve generation, and the cell culture supernatant was used for ELISA without dilution. This ELISA was used to measure FIX obtained from the following cell assay.

The cell-based VKOR inhibition assay was performed based on the method of Fregin et al. [4], with slight modifications. Briefly, HEK293T cells were seeded into 24-well collagen-coated multi-well plates and cultured to 70–80% confluency. Cells were transfected with the pcDNA3.1-C-(k) plasmid, which contained genes encoding human FIX and Norway rat (Rattus norvegicus) VKorc1, using Trans-IT293 Reagent (Takara-Bio). To minimize the influence of fetal bovine serum (FBS), we used reduced serum media (Opti-MEM, Thermo Fisher Scientific) supplemented with 4% charcoal-stripped and heat-inactivated FBS (SERANA, Brandenburg, Germany). Culture medium was collected 72 hr after the addition of vitamin K1-2,3-epoxide and warfarin dissolved in ethanol at concentrations of 10 μM and 0–500 nM, respectively. The concentration of ethanol in the culture medium was maintained at 0.1% throughout the experiment. Quantification of glaFIX in the culture medium was achieved by carrying out the sandwich ELISA without dilution. The level of secreted glaFIX was also measured using a RevoHem FIX measurement kit (Sysmex, Kobe, Japan), which is based on clotting activity. The plasmid DNA transfection was conducted in accordance with the Cartagena Protocol on Biosafety under the Convention on Biological Diversity and was approved by the Safety Committee for Genetic Recombination Experiments of School of Veterinary Medicine, Kitasato University (Approved number, V910-4767).

The concentration of glaFIX in the supernatant when no warfarin was added was found to be 106.3 ± 11.1 ng/ml, which was suitable for the inhibition assay, considering the LOQ. The level of secreted glaFIX decreased in a warfarin-concentration-dependent manner and inhibition curves could be drawn using nonlinear regression. Figure 2 shows the inhibition curves for ELISA and coagulation activity assays; the 100% relative activity value is taken as the concentration of glaFIX in the absence of warfarin. The kinetic values obtained were not significantly different between the two methods, with the ELISA and coagulation activity returning IC_{50} values of 26.8 ± 2.73 and 26.4 ± 2.12 nM, respectively (two-tailed unpairwise t-test, P<0.05). Thus, it can be concluded that the ELISA method has the same accuracy as the existing clotting-time-dependent assay.

We measured FIX levels in the culture medium by immunoblotting with the antibodies that were used in the ELISA. The polyvinylidene fluoride (PVDF) membrane was blotted with 4-fold diluted specimen and blocked with fish-gelatin solution. The following steps were performed using SNAP i.d. 2.0 Aspiration Immunoreaction System (Merck). After washing by aspiration with TBS-T, we applied anti-FIX antibody (GTX113689) diluted 3,500-fold with Western Blot Immuno Booster Solution 1 onto the membrane and incubated at room temperature for 30 min. After washing by aspiration with TBS-T, secondary antibody (anti-rabbit IgG HRP conjugated and preadsorbed, ab97080) diluted 5,000-fold with Western Blot Immuno Booster Solution 2 was added onto the membrane and incubated at room temperature for 10 min. Chemical luminescence detection was performed using HRP substrate (Clarity Wester ECL Substrate; Biorad) and the ChemiDocTM XRS Plus (BioRad). Figure 3A shows the bands detected by the FIX-specific antibody. Culture medium taken from VKORC1/FIX-cotransfected cells used in the inhibition assay showed two bands with almost the same molecular weight as the FIX standard, regardless of exposure to warfarin. Figure 3B shows these band intensities. The upper bands of the groups exposed to warfarin tended to be more intense compared to the control (exposed to vehicle only) although there were no significant differences in any of the combinations (Tukey-Kramer test, P>0.05). FIX is known to be synthesized as a precursor polypeptide which requires cleavage of the propeptide by Golgi membrane proteolytic protein furin and warfarin supplementation causes insufficient cleavage of it [16]. Thus, the lower band is considered a mature functional FIX, although the upper band seems to be similar molecular size to the FIX protein standard (STD). On the other hand, cell death was not observed after 72 hr of exposure to any concentration of warfarin. In addition, no band corresponding to FIX was detected in the mock, suggesting that FBS-derived/endogenous FIX secretion did not affect the assay.

Nearly 100 VKOR polymorphisms have been reported in rodents and humans, and many wildlife and pets are poisoned by rodenticides [9]. Therefore, identifying inter/intra-species differences in VKOR is important as it could direct the choice of rodenticide and reduce the risk to other animals. However, the IC_{50} value obtained from the traditionally used DTT-based assay is in the millimolar range, which is much higher than the concentration of ACC that is effective in vivo. Thus, the relevance of this assay to physiological conditions is questionable and so the choice of appropriate dosage for medicinal or rodenticide use is difficult. On the other hand, the IC_{50} obtained from cell-based assays is sub-nanomolar, which is close to the effective concentration in vivo, and so this is becoming the mainstream assay. There are several modified versions of the cellular VKOR assay, and the warfarin IC_{50} of wild-type VKOR obtained from these ranges from 1.2–24.8 nM (Table 1). These differences may be attributed to the use of different cell lines and FIX detection systems. In the present study, the IC_{50} of warfarin toward wild-type VKOR was similar to that reported by Fregin et al. [4] despite the fact that proteins from different species were studied (human or Norwegian rat VKORC1). However, because both studies used untreated 293T cells, the results can be considered to be reasonable. On the other hand, the lowest reported IC_{50} values are 1.2 and 3.48 nM, which are ten times lower than those obtained in the present study. One of the limitations of these cell culture VKOR assays is that the IC_{50} values of the same VKOR variant may differ depending on the experimental conditions. Therefore, when discussing IC_{50} values between different studies, it is better to evaluate the wild...
Fig. 2. Inhibition curves of warfarin. Secreted factor IX (FIX) activity data for HEK293T cells co-expressing human FIX with wild-type vitamin K epoxide reductase subunit C1 (VKORC1) of Norway rats in the presence of various warfarin concentrations (0, 25, 50, 100, 250, and 500 nM). The y-axis shows the percentage of activity in relation to the absence of warfarin (set as 100%). Data points represent mean values (n=4) and error bars show standard error of the mean. Nonlinear regression of warfarin inhibition curves and calculation of IC\textsubscript{50} values were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The IC\textsubscript{50} values are shown in Table 1. The two-tailed student’s t-test was performed using MEPHAS [18]. There were no significant differences in IC\textsubscript{50} values between the two assays (P<0.05). Abbreviations: ELISA, enzyme-linked immunosorbent assay; FIX, factor IX. IC\textsubscript{50}, 50% maximal inhibitory concentration.

![Graph showing warfarin inhibition curves and Table 1](image)

Table 1. Fifty % maximal inhibitory concentration (IC\textsubscript{50}) values for warfarin estimated by cell-based vitamin K epoxide reductase inhibition assay

| IC\textsubscript{50} ± SEM (nM) | VKOR origin | FIX measurement | Cell line | Reference |
|---|---|---|---|---|
| 24.8 ± 2.73 | Rattus norvegicus | Clotting time | 293T | This study |
| 24.6 ± 2.12 | R. norvegicus | glaFIX ELISA | 293T | This study |
| 24.7 ± 3.6 | Homo sapiens | Clotting time | 293T | Fregin et al. [4] |
| 12.1 ± 0.6 | H. sapiens | glaFIX–PC | 293 DKO | Shen et al. [14] |
| 3.48 | H. sapiens | glaFIX–PC | 293 DKO | Tie et al. [15] |
| 17.3 | H. sapiens | Clotting time | 293 DKO | Czogalla et al. [11] |
| 1.2 | R. norvegicus | Clotting time | 293EBNA | Müller et al. [8] |

The inhibitory effect of warfarin was measured by assessing clotting activity and by using a sandwich enzyme-linked immunosorbent assay, as shown in Fig. 2. IC\textsubscript{50} values were estimated using nonlinear regression with GraphPad Prism 9. These were compared with previously published warfarin IC\textsubscript{50} values against wild-type human/rat vitamin K epoxide reductase (VKOR) [1, 4, 8, 14, 15]. For measurement of secreted FIX, data collected using the system that measured the coagulant activity of FIX was designated “clotting time”, and that collected using the system involving the chimeric protein of the gla domain of FIX and protein C analyzed by ELISA was designated “glaFIX–PC”. The VKOR subunit C1 (VKORC1) and VKOR C1-like subunit (VKORC1L1) double knockout cell line was denoted as 293 (T) DKO. DKO, double knockout; ELISA, enzyme-linked immunosorbent assay; FIX, factor IX; PC, protein C; SEM, standard error of the mean; VKOR, vitamin K epoxide reductase.
type (WT) normalized ratio which is calculated by dividing the IC\textsubscript{50} values of the target VKORs by the IC\textsubscript{50} value of WT VKOR measured in each study rather than comparing the absolute IC\textsubscript{50} values. Shen et al. used this normalization as “the normalized warfarin resistance (nWar)” to compare their different sets of experiments and determined warfarin resistance when the ratio was 5-fold or greater [13]. This WT normalized ratio will reduce the impact of differences in experimental technique on the IC\textsubscript{50} value.

In conclusion, cellular assays that are based on inhibition of glaFIX secretion are an essential aspect in the assessment of ACCs. Previously, inhibition assays have required specialized equipment and reagents such as blood coagulation analyzers and glaFIXPC chimeric protein to quantify glaFIX. The sandwich ELISA assay presented here enables simple and direct quantification of glaFIX, which may facilitate the assessment of ACCs in general molecular biology laboratories.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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