Biochimica et Biophysica Acta 1840 (2014) 2382–2384

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Corrigendum

Corrigendum to "Determination of the warfarin inhibition constant Ki for vitamin K 2,3-epoxide reductase complex subunit-1 (VKORC1) using an in vitro DTT-driven assay" [BBAGEN (2013) 4202–4210]

Carville G. Bevans [a,1,2], Christoph Krettler [b,1], Christoph Reinhart [b,1], Hélène Tran [a], Katja Koßmann [a], Matthias Watzka [c], Johannes Oldenburg [c,*]

[a] Department of Structural Biology, Max Planck Institute of Biophysics, 60348 Frankfurt-am-Main, Germany
[b] Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, 60348 Frankfurt-am-Main, Germany
[c] Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, 53105 Bonn, Germany

1. Introduction

This article has been corrected at the request of the Authors. The authors have recently become aware that the published molar extinction coefficient used to determine all warfarin concentrations in our original report is incorrect [1]. The NCBI PubChem database (http://pubchem.ncbi.nlm.nih.gov, CID: 54678486, 54684598, 54689812, 54688261) states $\log_{10}(\varepsilon) = 3.05$ (equivalent to $\varepsilon = 1122$) at $\lambda = 287$ nm for warfarin free acid in ethanol and references this value to a peer-reviewed citation from the Hazardous Substances Data Bank (HSDB entry: WARFARIN CASRN: 81-81-2, http://toxnet.nlm.nih.gov/cgi-bin/sis/search/r?dbs+hsdb:@term+@rn+@rel+81-81-2) which, in turn, references the CRC Handbook of Chemistry and Physics, 60th ed., p. C-256 [2]. Additionally, the literature citation for Stahmann et al. (1944) referenced under the warfarin molar extinction coefficient entry in the CRC Handbook is also erroneous in that it does not report spectroscopic values for warfarin [3]. However, we have now measured the molar extinction coefficient for warfarin free acid in ethanol to be $\varepsilon = 9289\ M^{-1}\ cm^{-1}$ at $\varepsilon = 287$ nm (equivalent to $\log_{10}(\varepsilon) = 3.9680$). Specifically, we measured absorbance ($A_{287\ nm} = 0.39646 \pm 0.00013$(SEM), $n = 5$) of a solution comprising 42.68 μM warfarin free acid (6.58 mg, Warfarin PESTANAL® grade, racemic free acid, 99.9% pure, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) in ethanol (500 mL, ROTIPURAN® grade ethanol (99.8% pure), Carl Roth GmbH, Karlsruhe, Germany) using a Varian Cary 50 Bio UV/Visible Spectrophotometer with Cary WinUV software; Agilent Technologies Deutschland GmbH, Böblingen, Germany with a semi-micro Quartz SUPRASIL® spectroscopy cell with PTFE lid, light path 10 mm, B0631049; PerkinElmer, Rodgau, Germany. Thus, all warfarin concentrations in our original report must be corrected to values lowered by a factor of 8.2786 (i.e., the quotient of the measured to the previously reported molar extinction coefficients). Our determination of the warfarin free acid molar extinction coefficient in ethanol is in excellent agreement with the spectroscopic data of Karlsson et al. (2007) [4].

We would like to point out that, with the exception that all measured warfarin concentrations, IC_{50} and calculated K_i values should be corrected to exactly 8.2786-fold lower values than previously reported, all other results and conclusions stated in the original report remain unaffected and are correct to our best knowledge. For simplicity and accuracy in correcting the original report, the relevant text passages and Fig. 2 appear below in corrected form. Additionally, a corrigendum to the online Supplementary information has been made available at [http://dx.doi.org/10.1016/j.bbagen.2014.03.002]. We have notified the administrators of the NCBI PubChem and TOXNET (U.S. National Library of Medicine) databases, as well as the current publisher of the CRC Handbook (CRC Press, Taylor and Francis Group LLC), to effect correction of erroneous warfarin molar extinction coefficients.

In addition to correcting warfarin concentrations, further corrections to our original report include specific instances where values of pH 7.6, 225 nm, 248 nm and 278 nm are intended in lieu of the previously printed values pH 7.5, 266 nm, 249 nm and 271 nm, respectively (see following corrected text).

2. Materials and methods

2.1. Chemicals & reagents

Concentrations of vitamin K quinone (100 μM) and K > O (400 μM) stock solutions in ethanol were confirmed by UV absorption using molar extinction coefficients of 18,900 $M^{-1}\ cm^{-1}$ at 248 nm and 30,800 $M^{-1}\ cm^{-1}$ at 225 nm, respectively [5].

2.2. Preparation of ER membranes enriched for VKORC1

The VKORC1-enriched membranes were collected and centrifuged three times followed each time by resuspension using a tight-fitting

DOI of original article: http://dx.doi.org/10.1016/j.bbagen.2013.04.018.
* Corresponding author at: Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Sigmund Freud Str. 25, 53105 Bonn, Germany. Tel.: +49 228 287 5175; fax: +49 228 287 5176.
E-mail address: johannes.oldenburg@ukb.uni-bonn.de (J. Oldenburg).
[1] These authors contributed equally.
[2] Permanent address: Im Hermeshain 6, 60388 Frankfurt-am-Main, Germany.

http://dx.doi.org/10.1016/j.bbagen.2014.03.002

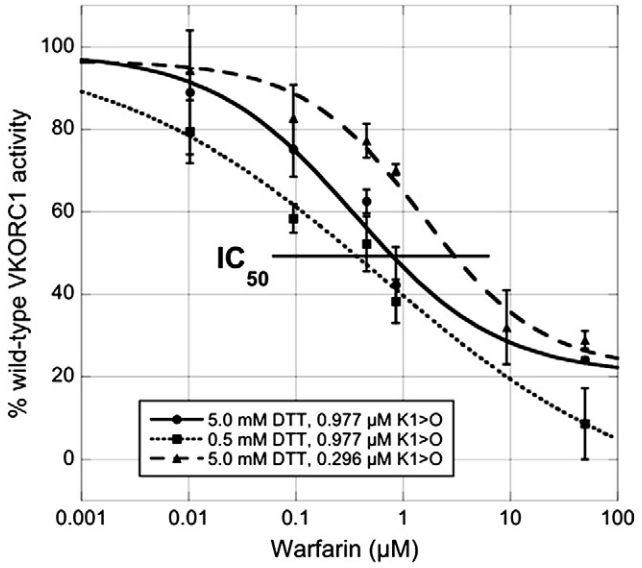

Fig. 2. Warfarin dose–response data for human VKORC1 measured using three distinct combinations of $K_1 > O$ and DTT substrate concentrations at pH 7.6. Mean VKOR activities for various warfarin concentrations at 5 mM DTT and 0.977 μM $K_1 > O$ (filled circles), 0.5 mM DTT and 0.977 μM $K_1 > O$ (open circles), and 5 mM DTT and 0.296 μM $K_1 > O$ (open squares); all data at pH 7.6 for N = 3 independent measurements. Bars indicate ± SEM. Points for each data set at zero warfarin concentration are valued at 100% activity and included in the curve-fitting calculations, but are not visible in the plot due to the logarithmic abscissa scaling. Best-fit four parameter logistical dose–response curves are shown for 5 mM DTT and 0.977 μM $K_1 > O$ (solid line, $R^2 = 0.978$, warfarin IC_{50} 748.6 nM), 0.5 mM DTT and 0.977 μM $K_1 > O$ (dotted line, $R^2 = 0.991$, warfarin IC_{50} 340.3 nM), and 5 mM DTT and 0.296 μM $K_1 > O$ (dashed line, $R^2 = 0.982$, warfarin IC_{50} 2.853 μM) data.

glass dounce homogenizer to exchange the membranes into storage buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 0.02% (w/v) NaN_3).

2.3. In vitro DTT-driven VKOR assay

We collected data for seven different warfarin final concentrations 0 nM, 10.3 nM, 95.1 nM, 458.6 nM, 856.3 nM, 9.271 μM, 49.88 μM; all concentrations determined by absorbance spectroscopy to values of four significant figures using $\varepsilon = 9289\ M^{-1}\ cm^{-1}$ at $\lambda = 287$ nm.

2.4. Warfarin dose–response curve fitting and IC_{50} value analysis

Final warfarin concentrations in the assay samples were 0, 0.01032, 0.09510, 0.4586, 0.8563 or 49.88 μM; for the dose–response data using 0.296 μM $K_1 > O$ and 5 mM DTT at pH 7.6, an additional final warfarin concentration of 9.271 μM was used to obtain additional data nearer to the apparent IC_{50} value to increase accuracy in curve fitting.

Half-maximum inhibitory concentrations for warfarin (IC_{50}) were calculated by solving the 4 parameter dose–response equation for the independent variable as a function of the dependent variable and substituting a dependent value of 50 since VKOR activities were normalized to 100% in the absence of warfarin inhibitor (see Supplementary methods and Table S1 of the Corrigendum to the online Supplemental information).

3. Results

3.1. Determination of Michaelis constants for $K_1 > O$ and DTT thiolate substrates

For various substrate concentrations ranging from 0.069 μM to 1.41 μM $K_1 > O$ and from 0.1 mM to 20 mM DTT at pH 7.6 (equivalent thiolate concentration range 2.2–449.3 μM), we made three independent measurements (N = 3) of VKORC1 enzymatic activity monitored as K_1 product produced during the linear phase of the VKOR assay reaction over 60 min.

Accordingly, data for $K_1 > O$ concentrations of 0.977 μM and 1.41 μM and for DTT concentrations of 0.5 mM and 1.0 mM at pH 7.6 were used for Michaelis constant determinations (Tables S2, S3 of the online Supplemental information). By Eisenthal & Cornish-Bowden direct linear plot analysis, the Michaelis constant for $K_1 > O$ ($K_{m,K1>O}$) for the human VKORC1 construct in *Pichia pastoris* ER membranes was 1.24 μM at a DTT concentration of 0.5 mM and pH 7.6 (equivalent to 11.23 μM thiolate, ~1/18-fold $K_{m,thiolate}$). The Michaelis constant for DTT thiolate ($K_{m,thiolate}$) for the same VKORC1 construct and expression system was 188.3 μM (equivalent to 8.38 mM DTT at pH 7.6) at a $K_1 > O$ concentration of 20 μM (i.e., >16-fold $K_{m,K1>O}$).

3.2. VKOR enzymatic activity of human VKORC1 is maximal between pH 6.0 and 7.0 and is not Ca^{2+}-dependent

The results presented in Fig. 1A show that there is no significant difference for DTT-driven VKOR activity in either the presence of 3.7 mM Ca^{2+} or 0.5 mM EGTA (i.e., in the complete absence of Ca^{2+}) for the human VKORC1 construct when substrate concentrations were 0.977 μM $K_1 > O$ and 0.5 mM DTT, pH 7.6 (equivalent to 11.23 μM thiolate).

3.3. Measured warfarin IC_{50} values for human VKORC1 vary with both $K > O$ and DTT substrate concentrations — estimates of the warfarin K_i for human VKORC1 based on IC_{50} values determined at various substrate concentrations

We measured warfarin dose–response data (10.3 nM to 49.88 μM warfarin) for DTT-driven VKOR assay conditions holding DTT thiolate concentration constant while varying $K_1 > O$ concentration and, alternatively, holding $K_1 > O$ concentration constant while varying DTT thiolate concentration. Accordingly, Fig. 2 (see corrected Fig. 2) shows measured warfarin dose–response data for three different selected assay conditions at pH 7.6 with either 5 mM DTT (circles; equivalent to 112.3 μM thiolate or ~0.6 $K_{m,DTT}$) or 0.5 mM DTT (squares; equivalent to 11.2 μM thiolate or ~0.06 $K_{m,DTT}$), both in the presence of 0.977 μM $K > O$ (equivalent to ~0.8 $K_{m,K1>O}$), and with 5 mM DTT in the presence of 0.296 μM $K_1 > O$ (triangles; equivalent to ~0.24 $K_{m,K1>O}$).

Calculated IC_{50} values for warfarin inhibition of VKORC1 enzymatic activity using 0.977 μM $K_1 > O$ were 0.7486 μM at 112.3 μM thiolate and 0.3403 μM at 11.23 μM thiolate, representing a 2.2-fold increase in measured IC_{50} value when DTT concentration was increased from 0.5 mM to 5.0 mM at pH 7.6. The difference in IC_{50} values we measured for these conditions is in excellent agreement with the 2.1-fold theoretical ratio we calculated for IC_{50} values obtained by substituting the experimentally applied substrate concentrations and measured K_m values into Eq. (1) (see corrected Table S4, Corrigendum to online Supplementary information). Alternatively, when we lowered the $K_1 > O$ substrate concentration, holding DTT thiolate concentration constant at 112.3 μM, we determined a warfarin IC_{50} value of 2.853 μM for an initial $K_1 > O$ concentration of 0.296 μM. Compared to the 0.7486 μM warfarin IC_{50} for 112.3 μM thiolate and 0.977 μM $K_1 > O$, this represents a 3.8-fold measured increase in IC_{50} value.

Substituting the experimentally determined IC_{50} values (0.3403 μM, 0.7486 μM, 2.853 μM) together with the corresponding $K_1 > O$ and thiolate substrate concentrations and Michaelis constants into Eq. (1) provides estimates of the VKORC1-intrinsic warfarin inhibitory constant (K_i) equal to 299.7, 318.1 and 696.8 nM, respectively (K_i mean value 438.2 ± 129.4(SEM) nM, K_i median value 318.1 nM, for the group of three independent determinations).

4. Discussion

4.1. Warfarin IC_{50} values determined for VKORC1 by the DTT-driven in vitro assay vary with substrate concentrations in a manner consistent with the bi bi ping-pong kinetic model and can be transformed to yield estimated values for the assay condition-independent K_i constant

Two of the three estimated warfarin K_i values were in close agreement (299.7 nM, 318.1 nM) when $K_1 > O$ concentration was held constant and DTT thiolate concentration altered, while the third estimate was somewhat greater (696.8 nM) when the $K_1 > O$ concentration was decreased while holding the DTT thiolate concentration constant (Table S4, online Supplementary information).

The fact that, of the two very similar K_i values obtained at constant $K_1 > O$ concentration, the 318.1 nM value for the greater DTT thiolate concentration is somewhat greater than the 299.7 nM value for the lower thiolate concentration also supports this notion as using the third assay condition with lower $K_1 > O$ concentration would be expected to further exacerbate the increased offset in measured IC_{50} and, hence, also in K_i value estimated by the Eq. (1) transformation.

In order to better define the fitted dose–response curve for this data series, we measured additional assay samples at a warfarin concentration of 9.271 μM, closer to the resulting IC_{50}.

Acknowledgment

The authors would like to thank Profs. Ian Nicholls and Bjorn Karlsson, Linnaeus University, Sweden, for technical advice.

References

[1] C.G. Bevans, C. Krettler, C. Reinhart, H. Tran, K. Koßmann, M. Watzka, J. Oldenburg, Determination of the warfarin inhibition constant Ki for vitamin K 2,3-epoxide reductase complex subunit-1 (VKORC1) using an in vitro DTT-driven assay, Biochim. Biophys. Acta 1830 (2013) 4202–4210.
[2] R.C. Weast (Ed.), CRC Handbook of Chemistry and Physics, 60th ed., CRC Press Inc., Boca Raton, Florida, USA, 1979.
[3] M.A. Stahmann, L.H. Graf, C.F. Huebner, S. Roseman, K.P. Link, Studies on 4-hydroxycoumarins IV esters of the 4-hydroxycoumarins1, J. Am. Chem. Soc. 66 (1944) 900–902.
[4] B.C.G. Karlsson, A.M. Rosengren, P.O. Andersson, I.A. Nicholls, The spectrophysics of warfarin: implications for protein binding, J. Phys. Chem. B 111 (2007) 10520–10528.
[5] M.J. Fasco, P.C. Preusch, E. Hildebrandt, J.W. Suttie, Formation of hydroxyvitamin K by vitamin K epoxide reductase of warfarin-resistant rats, J. Biol. Chem. 258 (1983) 4372–4380.