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Characterization of a Hemoglobin Adduct from Ethyl Vinyl Ketone Detected in Human Blood Samples

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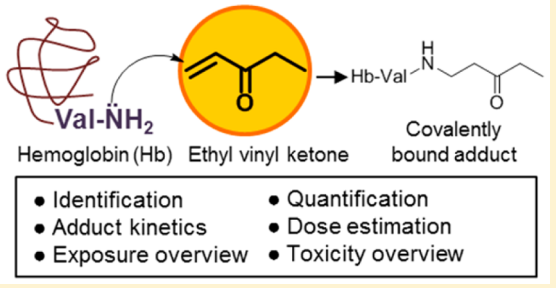
Supporting Information

ABSTRACT: Electrophiles have the ability to form adducts to nucleophilic sites in proteins and DNA. Internal exposure to such compounds thus constitutes a risk for toxic effects. Screening of adducts using mass spectrometric methods by adductomic approaches offers possibilities to detect unknown electrophiles present in tissues. Previously, we employed untargeted adductomics to detect 19 unknown adducts to N-terminal valine in hemoglobin (Hb) in human blood. This article describes the characterization of one of these adducts, which was identified as the adduct from ethyl vinyl ketone (EVK). The mean adduct level was 40 ± 12 pmol/g Hb in 12 human blood samples; adduct levels from acrylamide (AA) and methyl vinyl ketone (MVK) were quantified for comparison. Using L-valine *p*-nitroanilide (Val-*p*NA), introduced as a model of the N-terminal valine, the rate of formation of the EVK adduct was studied, and the rate constant determined to 200 $M^{-1}h^{-1}$ at 37 °C. In blood, the reaction rate was too fast to be feasibly measured, EVK showing a half-life <1 min. Parallel experiments with AA and MVK showed that the two vinyl ketones react approximately 2×10^3 times faster than AA. The EVK-Hb adduct was found to be unstable, with a half-life of 7.6 h. From the mean adduct level measured in human blood, a daily dose (area under the concentration−time-curve, *AUC*) of 7 nMh EVK was estimated. The *AUC* of AA from intake via food is about 20 times higher. EVK is naturally present in a wide range of foods and is also used as a food additive. Most probably, naturally formed EVK is a major source to observed adducts. Evaluation of available toxicological data and information on occurrence of EVK indicate that further studies of EVK are motivated. This study illustrates a quantitative strategy in the initial evaluation of the significance of an adduct detected through adduct screening.



INTRODUCTION

Humans are continuously exposed to electrophilic compounds/metabolites, both from exogenous and endogenous sources. Such exposures constitute risks for toxic effects due to the ability of electrophiles to form adducts with nucleophilic sites in proteins and DNA. Adducts, particularly to hemoglobin (Hb), have been employed for biomonitoring of exposure to the precursor chemicals using methods for mass spectrometric (MS) analysis.[1,2] So far, these studies have focused on adducts from exposure to one or a few chemicals occurring in occupational environments, tobacco smoke, food, etc. In many studies, adduct levels from the studied compounds have also been observed in control subjects, which has led to the detection of background exposures to a few carcinogens.[1,2] Such observations have demonstrated that the analytical methods are sufficiently sensitive to detect internal exposure to electrophiles in the general population. This fact is explored in adductomic approaches where a priori unknown adducts to blood proteins and DNA are screened for by using MS techniques.[3,4]

As previously reported, a strategy has been developed to screen by LC-MS/MS for unknown adducts to N-terminals in Hb in human blood.[5] Nineteen unknown adducts, with mass spectrometric characteristics similar to those of several identified adducts previously studied, were observed in blood samples from smokers and nonsmokers. Adduct screening was based on the FIRE procedure, a modified Edman degradation reaction where adducts to N-terminals in Hb are selectively detached using the reagent fluorescein isothiocyanate (FITC) and analyzed as fluorescein thiohydantoin (FTH) derivatives.[6] Modified Edman degradation procedures are well established for Hb adduct measurements.[7] The FIRE procedure has been developed for LC-MS analysis of adduct derivatives and is relatively fast with regard to workup and analysis.[6] Derivatization with Edman reagents has been shown to give detachment of N-substituted N-terminal valines in Hb with high specificity through a gem-dialkyl effect which occurs when the valines are N-substituted.[8]

Advantages of Hb as a monitor molecule for electrophiles is the accumulation of adducts over a well-defined lifetime and the high concentrations of Hb in blood. For many electrophiles, the major sites in Hb for adduct formation are cysteine-S, the ring-nitrogens of histidine, and the NH_2 group of N-terminal valine.[1] Other sites where adducts might form are the carboxylate groups of aspartic and glutamic acid and the C-

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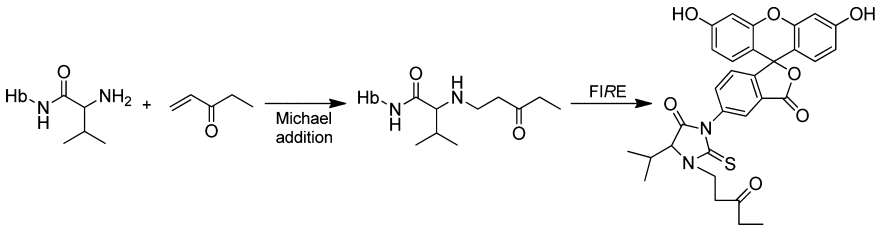
Scheme 1. Formation of N-Terminal EVK Adducts in Hb, Followed by Derivatization through the FIRE Procedure[a]



[a]EVK reacts with the amino group of Val through Michael addition and yields an EVK adduct (*N*-pentyl-3-one modification). The derivative, EVK-Val-FTH, is analyzed using LC-MS/MS.

terminal amino acid as well as to serine, threonine, tyrosine, lysine, arginine, methionine, and tryptophan residues. The effective enrichment and the sensitivity of the developed analytical methods based on modified Edman degradation make adducts to N-terminal valine suitable for studies of low adduct levels in humans. Another advantage with regard to unknown adducts is that NH_2-substituted amino acids are not prone to undergo misincorporation during the in vivo synthesis of proteins as has been shown for N-substituted N-terminal valine in Hb.[9]

The aim of the study was to use the previously collected adductome data to identify one of the unknown adducts and to identify the probable precursor electrophile, and in the next step, to make a quantitative characterization of the adduct to estimate the doses of the precursor electrophile in humans. Furthermore, a review of the literature together with the generated data, aiming at an evaluation of the detected background exposure with regard to possible sources and with regard to relevance for further toxicological studies, was also done.

The studied adduct is identified and corresponds to an adduct from ethyl vinyl ketone (EVK; 1-penten-3-one). EVK is an α,β-unsaturated ketone and forms adducts by Michael addition, Scheme 1. The analyte formed by derivatization with the FIRE procedure is fluorescein-5-[4-isopropyl-3-(pentyl-3-one)-2-thioxo-imidazolidin-5-one] (EVK-Val-FTH).

This is our first follow-up study of unknown adducts detected in the adductomics screening and as such is a model study to develop procedures to characterize and evaluate the significance of an unknown adduct detected by adductomics.

MATERIALS AND METHODS

Caution: *Fluorescein isothiocyanate (FITC), acrylamide (AA; prop-2-enamide), methyl vinyl ketone (MVK; 1-butene-3-one), and ethyl vinyl ketone (EVK; 1-pentene-3-one) are hazardous and should be handled with care.*

Chemicals. The analytical standards for the adduct derivatives of AA and MVK, fluorescein thiohydantoins (FTHs), formed from N-substituted valine/d_7-valine, were synthesized as described earlier[6,10] and stored in acetonitrile (ACN)/H_2O (1:1, v/v) at −20 °C until use. The following FTH standards were used: fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (AA-Val-FTH), fluorescein-5-[4-d_7-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (AA-d_7-Val-FTH), fluorescein-5-[4-isopropyl-3-(butyl-2-one)-2-thioxo-imidazolidin-5-one] (MVK-Val-FTH), and fluorescein-5-[4-d_7-isopropyl-3-(butyl-2-one)-2-thioxo-imidazolidin-5-one] (MVK-d_7-Val-FTH). Fluorescein-5-isothiocyanate (Isomer I, Reagent grade) was obtained from Karl Industries (Aurora, OH, USA). EVK (≥95%, food grade), MVK (99%), AA (99%), and L-valine *p*-nitroanilide hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and obtained from Sigma-Aldrich.

Equipment for Sample Preparation. Solid phase extraction (SPE) cartridges Oasis Max (3 cc, 60 mg, 60 μm; mixed mode anion exchange) were obtained from Waters (Milford, MA, USA). A Thermomixer comfort and a 5804 R centrifuge with rotor F-45-30-11 (Eppendorf Nordic, Denmark) were used for workup of blood samples derivatized with FITC. The Hb analyzer (Hb 201+) was obtained from HemoCue (Ängelholm, Sweden).

Study Population/Blood Samples. Blood samples from six smokers and six nonsmokers were earlier collected with ethical approval from the Regional Ethical Review Board in Stockholm (nr 96-312). The samples collected in heparinised tubes were separated into red blood cells (RBCs) and plasma. The RBCs were washed three times with 0.9% (w/v) sodium chloride and then lysed by the addition of distilled water before storage in a freezer (−20 °C). Human whole blood used as a reference sample and for kinetic experiments was obtained from Komponentlab at Karolinska University Hospital (Stockholm, Sweden). Bovine blood was used for calibration samples, and for this blood sample, the RBCs were separated from the plasma and washed as described above. Blood from different species was used as control samples. Bovine, rabbit (*Oryctolagus cuniculus*), and guinea pig (*Cavia porcellus*) whole blood samples (all with citrate) were obtained from Håtunalab AB (Bro, Sweden). Blood samples from mice (*BalbC*) were collected with approval from the Uppsala Ethical Committee on Animal Experiments (application C322/12).

Sample Preparation. The Hb adducts were analyzed according to the FIRE procedure (the TradeMark belongs to Adduct Analys AB, Skolvägen 18, Enebyberg, Sweden).[6] Prior to derivatization, the Hb content was measured in the samples. The individual blood samples of hemolyzed RBCs (0.25 mL) were derivatized with FITC (5 mg), while mixed (37 °C at 750 rpm) overnight (total derivatization time 18 h). Deuterium-substituted reference analytes for AA and MVK adducts, AA-d_7-Val-FTH and MVK-d_7-Val-FTH, were added to the blood samples as internal standards (5 pmol/sample) after the derivatization. Proteins were precipitated with ACN (1.4 mL), the samples were centrifuged (10 min at 11 000 rpm), the supernatants alkalized with 0.5 M ammonium hydroxide (25 μL), and transferred to SPE columns (mixed mode anion exchange). The columns were washed with ACN, H_2O, and 0.5% (w/v) cyanoacetic acid in H_2O prior to elution of the analytes with 0.25% (w/v) cyanoacetic acid in ACN (1.4 mL). The samples were evaporated to dryness using air and redissolved in H_2O/ACN (100 μL, 3:2 v/v) prior to the LC-MS/MS analysis.

Liquid Chromatography−Mass Spectrometry. The LC-MS/MS system consisted of a Shimadzu Prominence LC 20 system (Shimadzu Corp., Kyoto, Japan) interfaced to an API 3200 Q-trap instrument with a TurboIonSpray interface (ESI), from AB Sciex (Concord, ON, Canada). A Discovery HS C18 column (3.0 μm, 2.1 mm × 150 mm), with a Discovery HS C18 guard column (3.0 μm, 2.1 mm × 20 mm) (Supelco Analytical, Bellefonte, PA, USA), was used for chromatographic separation. The mobile phase consisted of A, 0.1% (v/v) formic acid in H_2O/ACN (95:5, v/v), and B, 0.1% (v/v) formic acid in H_2O/ACN (5:95, v/v). A gradient was applied, starting from 20% B and increasing to 100% B in 25 min and kept at 100% B for 5 min before re-equilibrating the column with 20% B for 5 min prior to the following injection. The injection volume was 20 μL, and the flow rate was 120 μL/min. The MS instrument settings were the same as those described previously.[5]

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A total of eight MRM transitions were monitored. For the AA and MVK adduct analytes, one transition was used for each analyte (m/z 560.1 to 517.1 and m/z 559.1 to 516.1, respectively) and one for the respective deuterium-substituted internal standards (m/z 567.1 to 517.1 and m/z 566.1 to 516.1, respectively). For the EVK adduct analyte, EVK-Val-FTH, four transitions were monitored (m/z 573.1 to 445.1, m/z 573.1 to 460.1, m/z 573.1 to 489.1, m/z 573.1 to 530.1) to ensure the identity of the new analyte monitored. The dwell time for each MRM transition was 30 ms. To obtain fragmentation patterns of EVK, m/z 573.1 was monitored in product ion scan mode, using the ion trap detector (collision energy 60 V).

Quantification/Calibration Curve and Control Samples. Calibration curves were established using samples that were processed and analyzed the same way as the blood samples in the study, as described above. Separate calibration curves were made for each of the three studied analytes using the FTH standards of the adducts and internal standards. For AA and MVK, their respective deuterium-substituted internal standards were used. Such a specific internal standard was not available for EVK, and in this case, MVK-d_7-Val-FTH was used. Calibration samples were prepared using 250 μL portions of a sample of bovine blood (Hb 148 g/L) as matrix (with background level of EVK adducts below the limit of detection). Each of the two internal standards (5 pmol) and the calibration standards (synthesis described below) (diluted in ACN/H_2O 3:2) were added during the sample preparation immediately after derivatization; see the section on Sample Preparation. The concentrations used for the calibration samples (n = 13) correspond to adduct levels approximately 0−10 000 pmol/g Hb and cover both the area of adduct levels in background samples and the higher levels reached from incubations of blood with the precursor electrophilic compounds. The m/z 573.1 to 530.1 MRM transition was used for quantification of EVK-Val-FTH. The calibration curves for the quantification by MS/MS analysis were established from the ratios between areas of the analyte and internal standard versus added amount of analyte per sample.

Bovine, rabbit, mouse, and guinea pig whole blood (250 μL) samples were used as control samples and derivatized and analyzed using the same procedures as those described above.

Synthesis of EVK-Val-FTH as a Reference Compound. The synthesis of the reference compound EVK-Val-FTH was done according to von Stedingk et al.,[6] with minor modifications. L-Valine (33 mg, 282 μmol) was dissolved in 0.275 M NaOH (1 mL, 275 μmol), and EVK (16.5 mg, 196 μmol) was added, and the reaction solution was mixed overnight (30 °C at 500 rpm) to generate *N*-pentyl-3-one-valine. ACN (680 μL) was added, followed by the addition of FITC (70 mg, 180 μmol) in dimethylformamide (420 μL). The solution was left for derivatization overnight (30 °C at 500 rpm). The derivatization was terminated by the addition of 2.7 M HCl (150 μL, 405 μmol).

The desired product was purified using semipreparative HPLC-UV. A Hichrom C18 column (5.0 μm, 10 mm × 250 mm) was used for the chromatographic separation. Isocratic mode was used, with the mobile phase ACN/H_2O (7:3). The injection volume was 100 μL, and the flow rate was 4 mL/min. The wavelength used was 268 nm. Two major peaks were observed, the latter eluting peak was identified as EVK-Val-FTH. The peak corresponding to EVK-Val-FTH was collected, and the purity of the fraction was confirmed by HPLC-UV (at 268 nm) and LC/MS (full scan in positive mode, other settings and parameters are as in the Liquid Chromatography Mass Spectrometry section above). Most of the solvent was removed by rotary evaporation, and the product was then dried in vacuo to yield 18 mg (31 μmol, 16% yield) of the desired product as a solid, orange colored, compound.

The ^{1}H NMR spectrum, recorded on a Bruker instrument, of the synthesized reference analyte was as follows (for the FTH part of the molecule the same annotation as used by Rydberg et al.[11] was used; for the "R group" (alkyl moiety of EVK modification), C1′−C5′ were used):

^{1}H NMR (500 MHz, d_3-ACN): δ ppm 0.94 [d, J = 6.9 Hz, 3 H, CH_3 (γ)], 1.01 [m, 3 H, CH_3 (C5′)], 1.24 [d, J = 7.3 Hz, 3 H, CH_3 (γ')], 2.50 [m, 2 H, CH_2 (C4′)], 2.54 [td, J = 6.9, 3.5 Hz, 1 H, CH (β)], 2.81−3.08 [m, 2 H, CH_2 (C2′)], 3.76 [dt, J = 14, 7.2 Hz, 1 H, (C1′)], 4.25 [d, J = 3.5 Hz, 1 H, CH(α)], 4.29−4.36 [m, 1 H, (C1′)], 6.75 [m, 2 H, xanthene-H], 6.85 [m, 2 H, xanthene-H], 6.92 [br s, 2 H, xanthene-H], 7.32 [dd, J = 8.2, 0.63 Hz, 1 H, C_7−H], 7.67 [dd, J = 8.2, 1.9 Hz, 1 H, C_6−H], 7.95 [d, J = 1.6 Hz, 1 H, C_4−H]. Refer to Supporting Information for the spectrum.

Reaction Kinetic Experiments. *In Vitro Incubations of Blood.* Several experiments were performed where blood was incubated with the studied electrophiles, all carried out in a similar way, and are described below. AA, MVK, and EVK, diluted to different concentrations (1−50 mM) in ethanol (EtOH; to minimize risk of polymerization) were added to preheated (37 °C) whole blood samples and allowed to react by mixing at 37 °C and 750 rpm for different, defined time periods. To stop the reaction, the samples were put on ice. The samples were then centrifuged (4000 rpm, 4 °C, 10 min), and the plasma was removed. The RBCs were washed two times with equal volumes of cold 0.9% (w/v) NaCl followed by centrifugation, and were then lysed by the addition of an equal volume of distilled water. The Hb content was measured in each sample before 250 μL was taken for derivatization, workup, and analysis as described above.

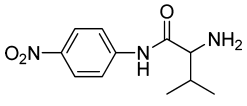
Reactivity of AA, EVK, and MVK toward N-Terminal Val in Hb. Incubations in whole blood were carried out for AA to estimate its reactivity toward N-terminal valine in Hb. To six preheated samples of human whole blood (1500 μL), AA (in 30 μL EtOH) was added to give concentrations of 0, 125, 250, 500, 750, and 1000 μM. The samples were incubated at 37 °C for 1 h before the reaction was stopped by putting the samples on ice.

A similar study showed that the reactions of EVK and MVK with N-terminal Val in Hb were too fast to be followed in whole blood. To slow down the reaction, blood was diluted ten times with water (190 μL blood in 1710 μL H_2O) and then incubated with EVK (at 5 μM initial concentration). Several identical samples were prepared and incubated simultaneously. Samples were taken every 15 min for a period of 2.5 h, and two additional samples were taken the following day at 21 and 24 h after the initiation of the reaction. To stop the reaction, the incubated samples were transferred to cold 0.9% (w/v) NaCl (5 mL) and cooled on ice. The samples were then centrifuged (4000 rpm, 4 °C, 5 min), and the supernatant, containing plasma and the NaCl solution, was removed, and the samples were washed with 0.9% (w/v) NaCl (5 mL), centrifuged, and the washing solution removed. Water was added to the washed erythrocytes, and the samples were then stored in a freezer (−20 °C). Prior to derivatization with FITC, the samples were thawed, and the Hb content was measured.

To follow the time-dependent decay of the formed N-terminal EVK adduct, human whole blood was incubated with EVK (25 μM) for 30 min and then put on ice. At this time, the reaction for the formation of the N-terminal EVK adduct was expected to be complete (based on the previous experiment). The sample was divided into multiple portions of 250 μL, which were mixed at 37 °C and 750 rpm. The reaction was terminated at specified times (0, 1, 2, 5, 9, 14, 18, 22, and 26 h) by putting the samples on ice. After 5 min on ice, the samples were put in a freezer (−20 °C). All of the samples were then thawed and derivatized with FITC at the same occasion, according to the procedure described above.

Reactivity of AA, EVK, and MVK toward L-Valine p-Nitroanilide. The reactivities of AA, EVK, and MVK toward L-valine *p*-nitroanilide (Val-*p*NA) (Scheme 2) were measured. Pseudo-first-order conditions were used with the individually tested electrophiles in large excess compared to the concentration of Val-*p*NA. Reactions were performed in 10 mM phosphate buffer, pH 7.4, at 37 °C and 750 rpm. AA, EVK,

Scheme 2. L-Valine *p*-Nitroanilide



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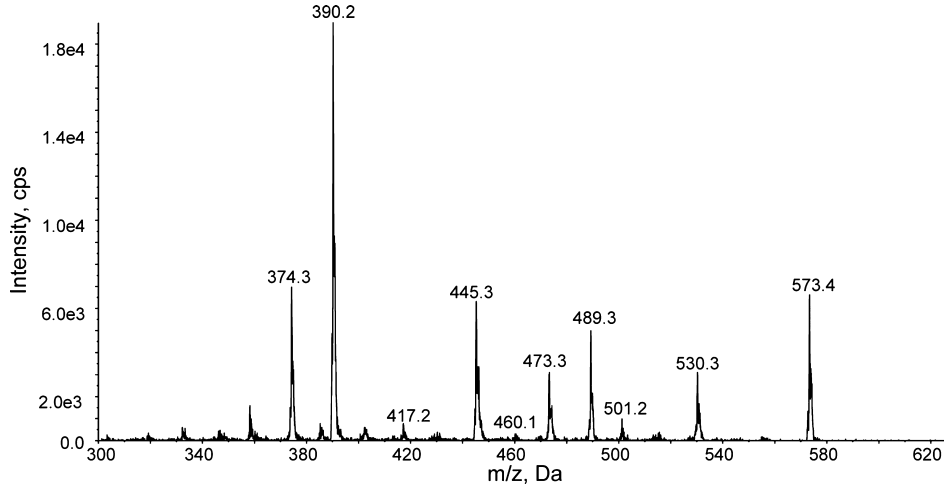

Figure 1. Fragmentation pattern of EVK-Val-FTH, m/z 573 ($[M + H]^+$), obtained from one of the calibration samples (bovine blood as matrix).

and MVK, dissolved in buffer, were added individually to Val-*p*NA in buffer (preheated to 37 °C). The total volume of the reaction mixtures was 1.5 mL. Val-*p*NA, 28.5 nmol, was used in the studies of all three electrophiles but with different concentrations of the electrophiles; 28.5 μmol (1000-fold excess) of AA and 570 nmol (20-fold excess) each of EVK and MVK were used. Aliquots of the reaction mixtures were injected on the LC-MS coupled to a UV detector, at specific time intervals, 10 times (15 times for AA), over 3 h periods.

Separation of unreacted Val-*p*NA and N-substituted Val-*p*NA (with adducts) was done in isocratic mode with the mobile phase H_2O/ACN (3:1, v/v) and 0.1% (v/v) formic acid, for the reaction with EVK and MVK; and the mobile phase H_2O/ACN (4:1, v/v) and 0.1% (v/v) formic acid for the reaction with AA. An Ascentis Express C18 column (2.7 μm, 2.1 mm × 150 mm) (Supelco Analytical, Bellefonte, PA, USA) was used. The injection volume was 20 μL, and the flow rate was 0.12 mL/min. Analysis time was 8 min. The wavelength used was 310 nm, and for the MS detection, selected ion monitoring (SIM) mode was used with the ions monitored being m/z 238.1 (Val-*p*NA), 308.1 (MVK-Val-*p*NA), 309.1 (AA-Val-*p*NA), and 322.1 (EVK-Val-*p*NA). The following instrument settings, optimized for the detection of Val-*p*NA, were used for the MS: declustering potential 30 V, entrance potential 4 V, ion source gas 1 (N_2) 20 arbitrary units (au), ion source gas 2 (N_2) 15 au, curtain gas (N_2) 20 au, ion spray voltage 5500 V, and vaporizing temperature 450 °C. The analysis of processed samples was performed in positive ion mode with unit resolution (0.6−0.8 Da wide peaks). All samples were measured both in UV and with MS, and the UV peak areas were used for quantification. A calibration curve of eight concentrations of Val-*p*NA in buffer was prepared for quantitative determination of the formed products, assuming that the UV absorption does not change significantly for Val-*p*NA modified with electrophiles at the Val nitrogen. The concentrations used for the calibration curve were 0.6−38 μM (n = 7). To generate the calibration curve, peak areas from the UV detection were plotted against the added concentrations of Val-*p*NA.

Calculations. *Calculation of Second Order Rate Constants for Adduct Formation.* Measurements of Hb adducts were originally developed to enable determinations of dose in vivo of short-lived electrophilic compounds/intermediates.[12,13] The rate of formation, v, of reaction products (adducts), RY, in the reaction of an electrophilic compound, RX, with a nucleophilic compound, Y, is determined by the second order rate constant, k_Y, for the reaction and by the concentrations of RX and Y according to

$$v = \mathrm{d}[RY]/\mathrm{d}t = k_Y \times [RX] \times [Y] \quad (1)$$

This equation was used to calculate k_Y (in $M^{-1}h^{-1}$) for the reaction of AA with Hb-Val in blood and for the reactions of EVK, MVK, and AA with the nitroanilide from the initial rates of adduct formation (in M h^{-1}) and the initial concentrations of the reactants (in M). Alternatively, the unit mol/g Hb per Mh may be used for the rate constant of the reaction with Hb.

Calculation of Dose (AUC) in Blood in Vitro. Given that the degree of modification of Y is low (i.e., $[RY] \ll [Y]$, the normal case during exposure in vivo) eq 1 may be integrated to give

$$[RY]/[Y] = k_Y \times \int [RX]dt = k_Y \times AUC \quad (2)$$

Thus, the degree of modification, $[RY]/[Y]$, of a nucleophilic site in Hb, such as N-terminal valine, is proportional to the dose in the meaning area-under-the-concentration−time curve (AUC; expressed in M × h) of the precursor electrophile, RX.

The calculation of dose of an electrophilic agent from adduct measurements is dependent on knowledge about the stability of the adduct. Equation 2 was used to calculate the dose of EVK in whole blood from adduct levels measured after 30 min of incubation. At this time, the reaction is complete (EVK consumed), and the effect on adduct levels from degradation is negligible.

Calculation of Clearance of EVK in Blood in Vitro. The AUC calculated according to the above could be used in eq 3 to calculate a pseudo-first-order rate constant for disappearance, λ, in vitro of an electrophile (EVK in this case), through all parallel reactions in the blood:

$$AUC = [RX]_0/\lambda \quad (3)$$

where $[RX]_0$ denotes the initial concentration of RX.[12,13] The corresponding half-life, $t_{1/2}$, is calculated as 0.693/ λ.

Calculation of Dose (AUC) in Vivo. An estimation of the dose in vivo (in blood) of an electrophile from the corresponding Hb adduct level requires consideration of the exposure history and the extent of accumulation of adducts.[14] Stable Hb adducts accumulate over a period of time corresponding to the lifetime of the erythrocytes, t_{er}, to reach a steady-state adduct level ($[RY]/[Y]_{SS}$). The level depends on the daily adduct increments (a) (pmol/g Hb per day), according to eq 4:

$$\left(\frac{[RY]}{[Y]}\right)_{SS\ stable} = a\frac{t_{er}}{2} \quad (4)$$

Chemically unstable adducts approach a steady state more rapidly. The accumulation of these adducts is limited not only by the turnover rate of the erythrocytes but also by the inherent instability of the adducts. ($[RY]/[Y]_{SS}$ for unstable adducts can be calculated as follows (corrected formula according to Granath et al.):[14]

$$\left(\frac{[RY]}{[Y]}\right)_{SS\ unstable} = a\frac{1}{k_{el}}\left[1 - \frac{1 - e^{-k_{el}t_{er}}}{k_{el}t_{er}}\right] \quad (5)$$

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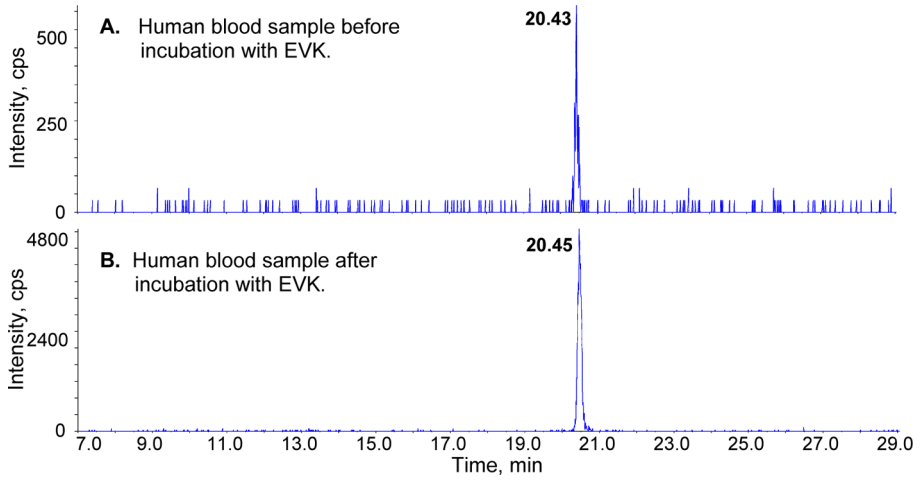

Figure 2. Mass chromatograms showing the transition m/z 573 to 445 of EVK-Val-FTH in (A) a background sample of human blood and (B) a human blood sample incubated with EVK.

where k_{el} is the first-order rate constant for elimination of adducts due to adduct instability. For unstable adducts with half-lives much shorter than the lifetime of Hb, the impact of t_{er} becomes negligible and $([RY]/[Y])_{SS}$ approaches a/k_{el}. The daily dose (AUC_d, e.g. in μMh/day) from exposure to electrophiles that form adducts that are stable (as from AA) or unstable (as from EVK) can then be calculated according to eq 2 using the daily adduct increment (a) for adduct level as obtained by eq 4 or (5), respectively, as given in eq 6.

$$AUC_d = a/k_Y \quad (6)$$

RESULTS

Identification of the EVK Adduct in Human Blood Samples. In a previous screening of Hb adducts to N-terminal Val with the FIRE procedure, an unknown adduct derivative with molecular ion $[M + H]^+$ m/z 573 was detected.[5] The adduct was tentatively proposed to correspond to EVK-Val-FTH. This was based on the m/z being 14 Da higher than the previously identified MVK adduct analyte, MVK-Val-FTH,[10] and that the compound eluted approximately 1.5 min later than MVK-Val-FTH during the 25 min gradient. This motivated an incubation experiment where EVK was added to a blood sample to form corresponding adducts. After derivatization with FITC, the incubated sample yielded a peak with the same retention time as that observed for the unknown adduct in background human blood samples, but with much higher intensity. The produced EVK analyte showed the same MS fragmentation pattern as the unknown compound. This strongly indicated that the identity of the unknown compound $[M + H]^+$ m/z 573 was the adduct analyte corresponding to EVK (pentyl-3-one adduct to the N-terminal Val amino group). To confirm the identity, EVK-Val-FTH was synthesized as a reference that was verified by ^{1}H NMR (shown in Supporting Information). The MS fragmentation pattern of EVK-Val-FTH is presented in Figure 1. Proposed structures of the observed fragments of highest abundance are found in the Supporting Information. The identity of the studied analyte from human blood was then verified as EVK-Val-FTH through agreement in MS analysis with the reference compound. Chromatogram of EVK-Val-FTH from an incubated sample and from a background sample are compared in Figure 2, and corresponding MS fragmentation patterns of the analyte in these samples and the synthesized reference are compared in Table 1.

Table 1. Comparison of Fragmentation Patterns of EVK-Val-FTH for (A) the Synthesized Reference Compound, (B) the Background Analyte, and (C) the Analyte in an Incubated Blood Sample

		relative intensity of fragments m/z (%)							
sample	Rt (min)	390	445	460	473	489	501	530	573
A[a]	20.58	100	33	2	16	26	5	16	36
B[b]	20.55	100	32	3	16	30	3	20	42
C[c]	20.56	100	32	2	16	23	3	16	31

[a]Synthesized reference compound added to bovine blood with no detected analyte background (calibration sample). [b]Background human blood sample (nonsmoker). [c]Human blood sample with low analyte background incubated with EVK (incubation concentration 5 μM, 15 min).

In Vitro Studies of Rate of Formation and Disappearance of EVK Adducts. To investigate what an observed adduct level to a site in human Hb means with regard to daily AUC (AUC_d) in blood of a precursor electrophile, one of the parameters that should be known is the second-order rate constant for the reaction of the electrophile toward that site in Hb at physiological conditions. Therefore, the rate constant for adduct formation toward N-terminal valine in Hb was sought for EVK. For comparison, AA and MVK were studied in parallel. Primarily, this was attempted by incubation of whole blood with the respective electrophile at different concentrations. The rate constant for the reaction of AA was determined in this way by plotting the quantified adduct levels (pmol/g Hb) for each incubated sample against the dose as μMh (the incubation concentration of AA is approximately constant during the incubation time[15] because of its relatively low reactivity). The measured rate constant ($k_{HbVal\text{-}AA}$) was 4.1 pmol/g Hb per μMh in accordance with previously published data.[16] For EVK and MVK, the reaction with N-terminal Val in Hb was found to be too fast to practically measure in whole blood. Therefore, an incubation experiment with EVK was conducted in diluted blood to slow down the reaction rate. However, it was clear that the maximum EVK-adduct level was reached already by 15 min, the first time point of measurement, which thus meant that the experiment did not allow a calculation of the reaction rate constant and that an alternative approach was required. Interestingly, the two samples taken at

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Chem. Res. Toxicol. 2015, 28, 2120−2129

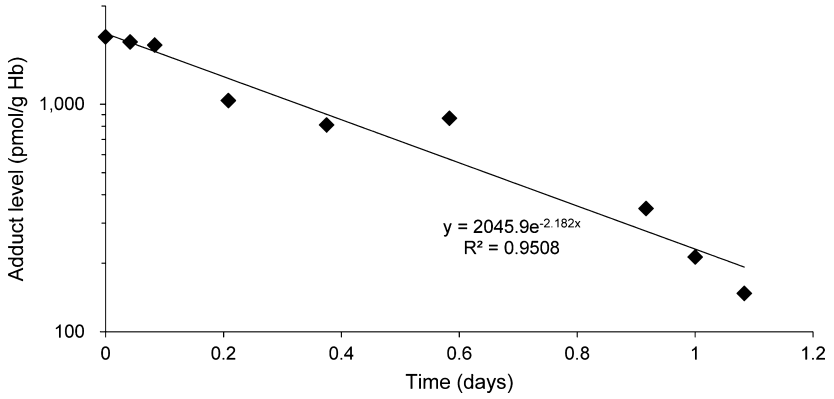

Figure 3. Decay over time of formed EVK adducts at 37 °C. The exponential function fitted to the data was used to estimate the rate of adduct decay ($t_{1/2}$ = 24 × (0.693/2.182) h = 7.6 h).

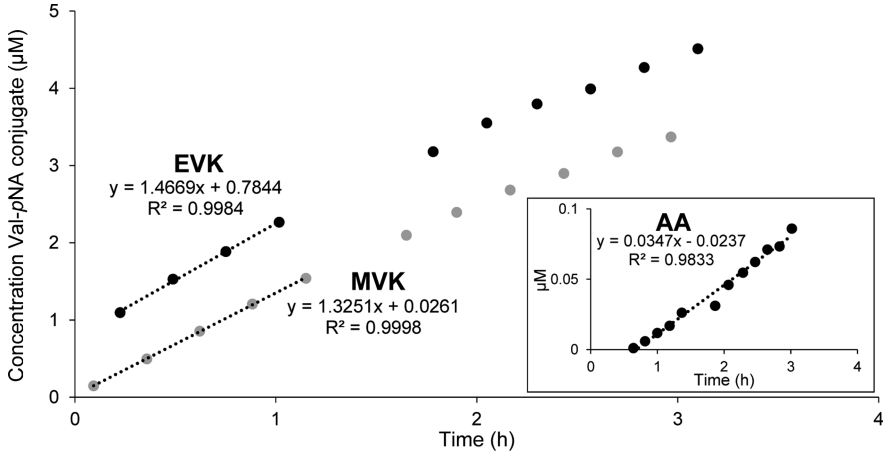

Figure 4. Formation of valine *p*-nitroanilide conjugates with EVK, MVK, and AA (insert) versus time of reaction, measured by HPLC-UV. The data points below 1.5 h (linear regressions in figure) were used to calculate the second-order rate constants (in $M^{-1}h^{-1}$) for the reactions of EVK and MVK.

the end of the experiment, at 21 and 24 h, exhibited significantly decreased adduct levels, indicating an instability of the EVK-Val adduct. This motivated a further experiment to study the rate of disappearance of the formed adducts, which showed that the level of the EVK adduct decreased with a half-life of 7.6 h at 37 °C (Figure 3).

To obtain an estimate of the reactivity of EVK and MVK toward N-terminal Val in Hb, Val-*p*NA was used as a model, which made it possible to follow the reactions of the highly reactive EVK and MVK, as well as of the relatively slow reacting AA. The rates of formation of adducts from the three compounds are shown in Figure 4. For AA, all data (above LOQ) were used. For MVK and EVK, where the reaction with nitroanilide is fast, only the measurements during the first 1.5 h were used to estimate the initial rate of adduct formation. The rate constants for EVK, MVK, and AA for reaction with Val-*p*NA ($k_{Val\text{-}pNA}$), calculated according to eq 1 from the initial rates of reaction, were 200, 180, and 0.10 $M^{-1}h^{-1}$, respectively, which means that EVK is about 2 × 10^3 times more reactive than AA and possesses similar reactivity as that of MVK.

By using the number of moles in 1 g of Hb (6.25 × 10^{-5} mol), the reaction rate constant obtained for the reaction of AA with N-terminal Val in Hb (in pmol/g Hb per μMh) can be converted to $M^{-1}h^{-1}$ for a comparison with the values obtained with Val-*p*NA. The rate constant 4.1 pmol/g Hb per μMh for AA corresponds to 0.07 $M^{-1}h^{-1}$, close to the value obtained for reaction of AA with Val-*p*NA (0.10 $M^{-1}h^{-1}$). When a similar conversion is done for EVK and MVK, assuming the same relative reactivity of Val-*p*NA and N-terminal Val in Hb toward these compounds, the rate constants for Hb are estimated as $k_{HbVal\text{-}EVK}$ = 12 × 10^3 and $k_{HbVal\text{-}MVK}$ = 11 × 10^3 pmol/g Hb per μMh.

The extremely high reactivity of EVK obtained implies a fast disappearance of EVK during the reaction in blood. The rate constant (λ) (or half-life) of EVK in vitro in blood, at 37 °C, was calculated using the obtained data. The second-order reaction rate constant for EVK (cf. above), together with the adduct levels in Hb obtained at 30 min of incubation (when the reaction is assumed to be completed and the influence on EVK adduct levels from degradation is negligible), and the initial concentration of EVK were used in eqs 2 and (3). The calculation indicates a very short half-life for EVK of 0.3 min in blood in vitro under physiological conditions.

Quantification of Adduct Levels and AUC from EVK in Vivo. The levels of EVK-Val adducts in the 12 blood samples from smokers and nonsmokers were quantified using the calibration curve established from the synthesized standard (R^2 = 0.9990, n = 13). The adduct levels in the samples ranged from 25 to 68 pmol/g Hb, with an average adduct level 40 ± 12 pmol/g Hb, and showed no significant difference between the six smokers and six nonsmokers (p value for two-sided t test was 0.49) (Table 2). Limit of detection (LOD) and limit of

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Chem. Res. Toxicol. 2015, 28, 2120−2129

Table 2. Quantified Adduct Levels of the Three Analyzed Adducts (AA, MVK, and EVK) in Nonsmokers and Smokers

		nonsmokers (pmol/g Hb) (n = 6)		smokers (pmol/g Hb) (n = 6)	
adduct	rt (min)	range	mean ± SD	range	mean ± SD
AA	15.44	20−62	40 ± 15	75−231	146 ± 59
MVK	19.03	57−95	76 ± 16	29−91	63 ± 23
EVK	20.56	26−68	43 ± 14	25−53	38 ± 10

quantification (LOQ) were estimated to 5 and 15 pmol g/Hb, respectively, based on the noise level obtained with processed samples (LOD was set to 3 times the signal-to-noise (S/N) ratio level and LOQ 10 times the S/N). Adduct levels of AA and MVK were determined as well and are given in Table 2 for comparison. For AA, a significant difference in adduct levels was observed between smokers and nonsmokers, in agreement with previous reports (e.g. by Bergmark[17]).

Assuming that the observed EVK adducts in the so-called background human blood samples are true in vivo formed adducts, and not formed in vitro as an artifact, we calculated a corresponding daily dose (AUC_d) of 7 nMh of free EVK in blood. Equation 5 was applied for calculation of the daily adduct increment (a) from the measured steady-state adduct level ($([RY]/[Y])_{SS}$), and then eq 6 was used for the calculation of AUC_d from $k_{HbVal\text{-}EVK}$ and a. As a comparison, the mean background steady-state adduct level from AA in the nonsmokers corresponds to a daily dose of about 160 nMh (eqs 4 and 6).

One test to trace possible exposure sources of EVK was to study the occurrence of the EVK adduct in Hb in blood from a few other species to find out if it is due to an exposure specific for humans. The adduct was detected in all 12 human blood samples with a range of 25−68 pmol/g Hb. The adduct was detected in one of two analyzed bovine blood samples, the guinea pig blood, and all tested (n = 6) mouse blood samples, with levels around LOD, i.e., 5 pmol/g Hb, but was below LOD in the rabbit blood.

DISCUSSION

Identification and Quantification of the EVK Adduct in Human Blood. The identity of the compound with $[M + H]^+$ m/z 573 observed in human blood samples was confirmed to correspond to EVK-Val-FTH after comparisons with the synthesized reference compound and with blood samples incubated with EVK. Fragmentation patterns and retention times in the MS analyses matched for the different samples (Figure 2 and Table 1). The identification of the EVK adduct was straightforward due to similarity with the previously observed adduct from MVK.

The quantification, using the synthesized standard, of the EVK adduct levels (40 ± 12 pmol/g Hb) in the studied human blood samples agrees well with the preliminary quantification (52 ± 13 pmol/g Hb) in the same samples from the initial screening of adducts.[5] This demonstrates the applicability of the previously used semiquantification in the screening to get an estimate of the levels of unknown adducts before accurate quantification with the authentic standard could be performed. The measured levels of EVK adducts in smokers and nonsmokers are comparable with the levels of AA adducts in nonsmokers (Table 2). The mean adduct level of EVK, 40 pmol/g Hb, corresponds to a fraction of about 7 N-substituted valines per 10^7 N-terminal valines demonstrating the need for enrichment of adducts provided by the FIRE procedure.

Potential Sources for the Formation of the EVK Adduct. Following the detection of an unknown adduct in human blood, confirming its identity and quantifying the adduct level, the next step is to find the origin and sources of the measured adduct. Therefore, we have studied whether the EVK adduct could be detected in Hb from other species and searched for information on possible sources of EVK exposure in the literature. Certain control experiments were done to exclude artifact formation after collection of samples or during workup in our previous work concerning screening of unknown adducts.[5]

The EVK adduct levels were around LOD in blood from bovine, mouse, and guinea pig and below LOD in the rabbit sample while detected in all analyzed human blood samples. Previously, we have used bovine blood for control experiments. For this study we added rabbit, mouse, and guinea pig blood since the N-terminal regions in Hb of these species are more similar to the human Hb (at least the three first amino acids in the sequences from the N-terminal are identical in both the α- and the β-chains, respectively).[18] Bovine Hb only contains N-terminal Val in 2 of the 4 Hb chains, whereas human, mouse, guinea pig, and rabbit Hb contain N-terminal Val in all of the 4 chains. The occurrence of background adduct levels around LOD in these species shows that the EVK adduct is not unique for human blood.

It is known that EVK is a naturally occurring and a synthetic flavouring substance with significant human exposure from food and beverages. According to the US Food and Drug Administration (FDA), one major occurrence of EVK is in orange essence oil,[19,20] but EVK has also been identified as a key flavor component of soybeans and reverted soybean oils[21] and has been found in a large number of food products including black tea,[22] raw and cooked potatoes,[23,24] tomatoes,[25,26] grapes,[27] kiwi fruit,[28] banana, grapefruit juice, peach, fish oil, chicken fat, lovage leaf, endive, oysters, clam, and boiled and cooked beef.[29] EVK has also been found in buttermilk, and it has been hypothesized that it might be formed from linolenic acid, which is present in many common seeds, nuts, and vegetable oils.[30] The occurrence of EVK in this diversity of food items overall strongly indicates that EVK is formed in natural cellular processes. We have found no data suggesting that EVK could be formed as a Maillard reaction product. It is also associated with tobacco and has been identified as a component of tobacco smoke. The US National Toxicology Program (NTP) has reported EVK as a natural component of tobacco and an additive to some tobacco products.[31]

The concentrations of EVK in individual food items are estimated to be low, but EVK is present in orange juice and other frequently consumed food products. An estimation by the FDA of the exposure of EVK, which was based on the market disappearance level of 4.5 kg per year of synthetic EVK for flavouring purposes, amounts to 0.014 μg/kg bw per day and person.[19] The WHO similarly estimates the exposure to 0.0057 and 0.0020 μg/kg bw per day and person in Europe and USA, respectively, based on an annual production.[32] From the literature, it could be concluded that there are exposure to EVK through food, and although the concentrations are not known, the presence of naturally formed EVK in such a variety of food items might indicate much higher levels of exposures than the figures given by the FDA and WHO, corresponding to synthetic EVK added to food.

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Chem. Res. Toxicol. 2015, 28, 2120−2129

EVK could possibly be formed endogenously in human tissues during metabolism and other cellular processes. One possibility is the formation from linolenic acid, which has been suggested as a source of EVK in certain foods.[30] Another example is the formation of EVK as a metabolite from 1-penten-3-ol, which has been shown for *Drosophila melanogaster*.[33] This compound is also used as a flavouring and fragrance additive and found naturally in banana, orange juice, raspberry, and other commonly consumed food products.[34] A study done in the USA in 1982 found naturally occurring EVK, among other ketones, present in 4 of 12 breast milk samples from humans (no data on observed concentrations are available).[35]

Calculation of *AUC* of EVK from Adduct Levels and Rate Constants. The *AUC* in blood of the precursor electrophile could be calculated from a measured Hb adduct level and is a useful parameter for further toxicological/toxicokinetic evaluation. Assuming a constant exposure level over a long period of time, the *AUC* could be calculated from an adduct level if the rate constant of adduct formation and the stability of the formed adduct are known. Because of difficulties in measuring the rapid rate of the reaction of EVK with N-terminal valine in Hb, Val-*p*NA was introduced as a model of N-terminal valine for reaction-kinetic studies. Val-*p*NA allows reactions to be followed by UV without further derivatization. Measurement of the reaction rate toward Val-*p*NA showed a 2×10^3 times higher reactivity of EVK, as well as of MVK, than of AA (order of reaction rate: AA < MVK ≈ EVK). This rate constant of EVK was then used to estimate the second-order rate constant for the reaction of EVK with N-terminal Val in Hb. Furthermore, it was observed that the measured EVK adduct in Hb was unstable, with a surprisingly short half-life (7.6 h), which means that the steady state is rapidly attained.

The daily dose, AUC_d, in humans was estimated to 7 nMh EVK, using the adduct kinetic data and mean adduct level. This is about 20 times lower than the calculated AUC_d for AA of 160 nMh in nonsmokers from exposure from food (cf. Vikström et al.[16,36]). From the AUC_d, one could estimate the exposure dose from external exposure if the detoxification rate, volume of distribution, and bioavailability are known. Here, the instability of adducts (discussed below) further complicates the estimation approach. The measured short half-life of EVK in vitro in blood (0.3 min) suggests that the clearance of EVK through chemical reactions is fast also in vivo. Assuming, tentatively, that the in vivo half-life of EVK is of the same order of magnitude as that in vitro we can make a rough estimate of the exposure concentration (through uptake or production in vivo) in humans. This simplified estimation gives that the measured steady-state EVK adduct level would correspond to an exposure concentration which appears unrealistically high as compared to the estimates of possible uptake from known sources of EVK. This indicates that the in vivo half-life might be much longer (due to possible reversibility of its reactions; see below).

Adduct Pattern and Instability of Adducts from EVK. Compounds like EVK with an activated double bond are soft electrophiles that can react with nucleophilic sites in biomolecules, usually in the order S > *N* > O.[37] In addition to N-terminal Val, Cys-S in Hb would be a major reactive site for adduct formation in blood like, e.g., Cys-S in the Cys34 site in serum albumin and in glutathione (GSH).

The results from the in vitro study demonstrates that the formed EVK-Val adducts are not stable. It has been shown, for several Michael acceptors (α,β-unsaturated ketones and aldehydes), that Michael addition can be reversible, through so-called retro-Michael reactions.[38] Similarly, the EVK moiety might detach from the N-terminal Val in Hb by a beta-elimination mechanism (cf. Monks et al.[38]) and could then possibly form new adducts. Such reversibility of adducts could prolong the lifetime of EVK in vivo. To our knowledge, the reversibility of formation of adducts to N-terminals in Hb from Michael addition has not been reported earlier. Considering the structural similarities between EVK and MVK, it is likely that MVK-Val adducts exhibit similar instability.

In Michael addition, the electrophile is added to the nucleophile, resulting in a single covalent bond. The energy threshold for the electrophile moiety to detach from a Michael addition product and reform should be considerably lower than that for adducts formed from S_N2 reactions, e.g., from an epoxide or an alkyl halide, when there is ring opening or a leaving group (halide), respectively, upon adduct formation. This type of instability of adducts is therefore expected for Michael addition products, like those formed from the electrophiles studied in this article.

The reversible conjugation of GSH with Michael acceptors has been studied in some detail. The reverse reaction proceeds rapidly at basic pH (pH 8), but at low pH (pH 4), it becomes slow at room temperature and in water.[39] The reaction of Michael acceptors with GSH at physiological pH in general proceeds very quickly, and due to reversibility of the reaction, a fraction of the conjugate reforms the electrophile.[40] Examples of such compounds reacting reversibly is a metabolite (an open-chain acrylonitrile derivative) of the veterinary drug furazolidone[41] and ethacrynic acid.[42,43]

Toxicity of EVK. There is limited data on the toxicity of EVK in the literature. Eder et al. have published several studies on the genotoxicity of EVK and done comparisons with MVK.[44] According to their results both compounds are clearly mutagenic in the Ames test (*Salmonella typhimurium*). When comparing the two compounds, by Ames test and SOS Chromotest (using *E. coli*), the mutagenicity and genotoxicity are clearly higher for EVK than for MVK. When the reactions of EVK were investigated with nucleosides and 5′-mononucleotides, only products to deoxyguanosine and 2′-deoxyguanosine 5′-monophosphate could be isolated; two types of guanine adducts were identified, linear N7 adducts and cyclic 1,N^2 adducts.[45] The linear adducts are proposed to be formed by Michael addition of the activated double bond of EVK to N7 deoxyguanosine, and the cyclic adducts are formed by the addition to N1 followed by ring closure between N^2 and the carbonyl group of EVK. The cyclic adducts were the main reaction products and are discussed as potentially significant DNA adducts for the observed genotoxicity of EVK.

The National Toxicology Program (NTP) investigated short-term inhalation toxicity of EVK in male and female rats and mice.[46] On the basis of this study, EVK seemed to be a direct-acting upper respiratory tract irritant and having few systemic effects.[46] Qualitatively, the toxicity of EVK was found to be similar to that for MVK, although EVK was less potent. Studies of micronuclei in rats and mice showed negative outcome for genotoxicity.[31,46]

Several computational studies have considered the toxicity of EVK. Mulliner et al. performed predictions of Michael acceptor reactivity toward GSH for a large number of α,β-unsaturated ketones and aldehydes.[47] Of the compared ketones, EVK and MVK were the two most reactive, MVK, however being more reactive than EVK. Koleva et al. investigated structure−activity relationships for α,β-unsaturated carbonyl compounds and

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Chem. Res. Toxicol. 2015, 28, 2120−2129

concluded that EVK is more mutagenic than MVK.[48] This was suggested to be due to the increased lipophilicity with the longer chain length of EVK that may allow for better penetration through the cell membrane.[49]

Perspective on Observed EVK Adducts in Hb from Humans. This study illustrates a strategy for structural identification and quantitative characterization as the first-step in evaluation of the significance of an unknown adduct detected in an adductomics screening. The unknown adduct was identified as the pentyl-3-one modification formed from EVK. It was shown that the rate of adduct formation of EVK is very high and further that the adduct is unstable with a short half-life. Altogether, these data indicated that the daily *AUC* of EVK in humans is considerably lower than the background exposure of AA from food. Following the identification, the EVK-Val adduct has been regularly observed in screened human blood samples (>100 samples, unpublished) in parallel studies. Naturally formed EVK is probably a major exposure source and is known to occur in food. Our study indicates that the exposure is not specific for humans. Earlier studies have shown that EVK is genotoxic in vitro. Overall, the results motivate further studies to quantify exposure sources of EVK, aided by adduct monitoring, and studies of, e.g., toxicokinetics and genotoxic potency, to evaluate the observed internal exposure to EVK with regard to possible contribution to health risks. The results illustrate the importance of a quantitative characterization of an unknown adduct in the first evaluation of its significance. The study strengthens that adductomics screening is a useful approach for the detection of exposures to electrophiles.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00287.

^{1}H NMR spectrum of the synthesized reference compound, proposed structures of fragments, and plot for the calculation of $k_{HbVal\text{-}AA}$ (PDF)

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Notes

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■ ABBREVIATIONS

AA, acrylamide; ACN, acetonitrile; au, arbitrary units; AUC, area under the concentration−time curve; eq, equation; EtOH, ethanol; EVK, ethyl vinyl ketone; FITC, fluorescein isothiocyanate; FTH, fluorescein thiohydantoin; Hb, hemoglobin; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MVK, methyl vinyl ketone; RBC, red blood cells; SPE, solid phase extraction; Val-*p*NA, L-valine *p*-nitroanilide

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