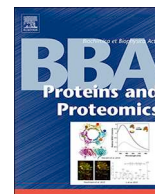Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Degradation of thymic humoral factor γ2 in human, rat and mouse blood: An experimental and theoretical study

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ABSTRACT

The degradation of the immunomodulatory octapeptide, thymic humoral factor γ2 (THF-γ2, thymoctonan) has been studied in whole blood samples from human, rat and mouse. The peptide, Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu, was shown to be rapidly degraded by peptidases. The half-life of the intact peptide was less than 6 min at 37 °C in blood from the three species tested. The main fragments formed from THF-γ2 were found to be Glu-Asp-Gly-Pro-Lys-Phe-Leu (2–8), Asp-Gly-Pro-Lys-Phe-Leu (3–8) and Glu-Asp-Gly-Pro-Lys (2–6) in human and in rat blood and 2–8 and 2–6 in mouse blood. Analysis of the time course of degradation revealed a sequential removal of single amino acids from the N-terminus (aminopeptidase activities) in a process that was apparently unable to cleave the Gly-Pro bond (positions 4–5 in the peptide) together with an independent cleavage of the Lys-Phe bond (positions 6–7 in the peptide) to release the dipeptide Phe-Leu. This behaviour and the effects of inhibitors showed the involvement of metallo-exopeptidases in the N-terminal digestion and a phosphoramidon-sensitive metallo-endopeptidase in the cleavage of the Lys-Phe bond. The degradation patterns in human blood were modelled in terms of the competing pathways involved approximating to first-order kinetics, and an analytical solution obtained via the method of Laplace Transforms. The half-life of THF degradation in whole rat blood sample was found to be significantly lower than in human or mouse.

1. Introduction

Thymic humoral factor γ2 (THF-γ2, thymoctonan; THF) is an immunomodulatory octapeptide that was originally isolated from calf thymus and shown to have the amino-acid sequence: Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu [1]. The peptide was shown to account for the observed effects of crude thymus extracts in stimulating T-cell functions [2,3] and interleukin 2 (IL-2) production [4]. It has also been shown to be of value for the treatment of some viral diseases [5], immune-insufficiency conditions [3] and as an adjunct to chemotherapy in cancer treatment [6].

Earlier work had shown the therapeutic value of the peptide to be limited by its relatively rapid metabolism in the blood. The half-life of THF in human plasma was reported to be about 12 min [7] and preliminary studies suggested that metabolism was faster in whole blood [8]. This led to the synthesis of analogues with greater stability [9], a process that could be aided by the identification of sites that are susceptible to degradative enzymes in blood. Since the behaviour in whole blood might be expected to be more complicated because of the contributions of cell-associated peptidases, an aim of the present study was to examine the rates of degradation product formation in human blood in vitro for comparison with the behaviour reported in plasma. As there may be species differences in THF metabolism the behaviour in mouse and rat blood was also studied for comparison with that determined in the human.

Attempts were made to model the behaviour of the system in terms of the competing pathways involved and the application of the Laplace transform [10] to the data obtained with human blood was shown to provide a more robust model of the system that could be applied to the degradation of other peptides and different deterministic systems.

2. Materials and methods

2.1. Peptides

THF-γ2 trifluoroacetate, [³H]-THF-γ2 trifluoroacetate labelled in the proline residue (specific activity 1.17 TBq/mmol, and [³H]-THF-2 trifluoroacetate, labelled in the C-terminal leucine residue (specific activity 1.75 TBq/mmol), synthesized as described by Fontana et al. (1996) [11] were kindly provided by Dr. P. Dostert (Pharmacia, Italy). Each had a radiochemical purity > 98%, as assessed by TLC and HPLC. The unlabelled peptides H-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH, H-Asp-Gly-Pro-Lys-Phe-Leu-OH, H-Gly-Pro-Lys-Phe-Leu-OH, H-Pro-Lys-Phe-Leu-OH, H-Leu-Glu-Asp-Gly-Pro-Lys-OH, H-Glu-Asp-Gly-Pro-Lys-OH were from the same source. The dipeptide H-Phe-Leu-OH and L-leucine were obtained from Sigma-Aldrich Chemical Co.

2.2. Reagents

Water was filtered through a Millipore Milli-U10 water purification system before use. Acetonitrile (hplc Grade) and hydrogen peroxide (30% m/m volume) were obtained from Sigma-Aldrich. Dichloromethane, methanol and propan-2-ol were from Romil Pure Chemistry (Cambridge, UK). Acetic acid and chloroform were from Avantar-BDH, UK. The Scintillation cocktail Ready Flow was from Beckman and Hionic Fluor and Soluene-350 were from Canberra Packard. The peptidase inhibitors amastatin, 1,10-phenanthroline amastatin and phosphoramidon were from Sigma-Aldrich and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was from Boehringer. They were dissolved in 137 mM phosphate-buffered saline (PBS), pH 7.5.

2.3. Animals and blood samples

Male rats (Wistar; weight 180–200 g) and male mice (Laca; weight 35–40 g) were used. The animals were fasted overnight, with free access to water, before blood was drawn. Animal manipulations were carried out with ethical approval from the Institute and according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). Rat and mouse blood was drawn from the heart, under halothane aesthesia, by cardiac puncture into heparinized tubes. Blood samples from three different rats or mice were used as separate samples in each study. Blood human was drawn into heparinized tubes from three healthy male blood donors.

2.4. Preparation and incubation of the samples

1 ml aliquots of blood were incubated in a water bath at 37 °C for 5 min and 20 μl of the [³H]-THF solution, corresponding to 800 nCi (30 ng of [³H]-THF labelled in the proline or 20 ng of [³H]-THF labelled in the C-terminal leucine), was added to give a final THF concentration of 93 nmol ml⁻¹. After vortex mixing (5 s) the samples were incubated at 37 °C in a shaking water bath. Samples were taken for analysis after 0, 1, 2, 5, 10, 15, 20, 30, 45 and 60 min. Each experiment was performed in triplicate.

2.5. Extraction of metabolites

The metabolism was allowed to proceed in air and finally stopped by addition of a mixture of 0.5 ml of ice-cold water and 2 ml of ice-cold acetonitrile followed by vortex-mixing and centrifugation at 1200*g* for 10 min. The upper phase was then transferred into another tube, while the corpuscular fraction was washed with 0.5 ml of water plus 1 ml of acetonitrile and centrifuged at 1200*g* for 10 min. The upper phase was collected and pooled with the upper phase previously obtained. The combined phases were extracted with 3 ml of dichloromethane, vortex-

Table 1
Chromatographic mobilities of THF-γ2 and derived peptides.

Peptide	Residues	R_f
H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH (THF-γ)	1–8	0.5
H-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH	2–8	0.3
H-Asp-Gly-Pro-Lys-Phe-Leu-OH	3–8	0.35
H-Gly-Pro-Lys-Phe-Leu-OH	4–8	0.41
H-Pro-Lys-Phe-Leu-OH	5–8	0.47
H-Phe-Leu-OH	7–8	0.87
Leu	1 or 8	0.58
H-Leu-Glu-Asp-Gly-Pro-Lys-OH	1–6	0.14
H-Glu-Asp-Gly-Pro-Lys-OH	2–6	0.05

mixed and centrifuged at 1200*g* for 10 min. The separated aqueous phase was submitted to radio-TLC analyses.

2.6. Chromatography

Separations were carried out on Silica gel plates (Merk-Millipore F254) 20 × 20 cm, 0.5 mm thickness. The mobile phase was water-methanol-acetic acid-chloroform (6:14:20:40 by volume). After drying the TLC plates in a current of warm air, the gel was scraped from 1 cm sections into scintillation vials containing 1 ml Soluene-350. 10 ml Hionic-Fluor was then added for liquid scintillation counting.

Under these conditions R_f values of THF and the synthetic peptides that could be derived from it were as shown in Table 1. These were used to identify the breakdown products from the zonal analysis of the blood incubations.

2.7. Effect of peptidase inhibitors

1 ml of human blood was pre-incubated in PBS (control) or in presence of varying concentrations of amastatin, 1–10 phenanthroline, phosphoramidon, or AEBSF for 30 min at 37 °C before labelled THF was added. The incubation was then continued for an additional 20 or 60 min at 37 °C before the reaction was terminated and the samples analysed as described above.

2.8. Calculations

Half-lives for THF decay in blood were calculated from the decay curves fitted to the exponential function A_0e^{-kt} where half-life, $t_{1/2} = (\ln 2)/k$. Curves were fitted using the program Prism 8 (GraphPad Software, LLC.) and values are expressed ± SEM. Apparent rate constants for the appearance of metabolite peptides were estimated by curve fitting the concentration-time curves for each substrate. The peptide degradation patterns for THF in human blood were fitted using the analytical functions described later (section 3.3).

3. Results

3.1. Peptide degradation patterns

THF was extensively degraded when incubated in human, mouse or rat blood. The time courses of degradation of the proline-labelled THF in rat and mouse blood are shown in Fig. 1A & B and those with human blood are shown in Fig. 2.

Studies with C-terminal leucine-labelled THF (not shown) showed the release of the dipeptide Phe-Leu (7–8), followed the same curve as that of peptide 2–6. As might be expected from the greater genetic heterogeneity, the behaviour of THF in blood samples from different human donors showed a greater variability than those from rat and mouse, as is evident from the larger errors with the human data (Fig. 2). However, these differences were largely reflected in the rates of degradation rather than the nature of the products formed.
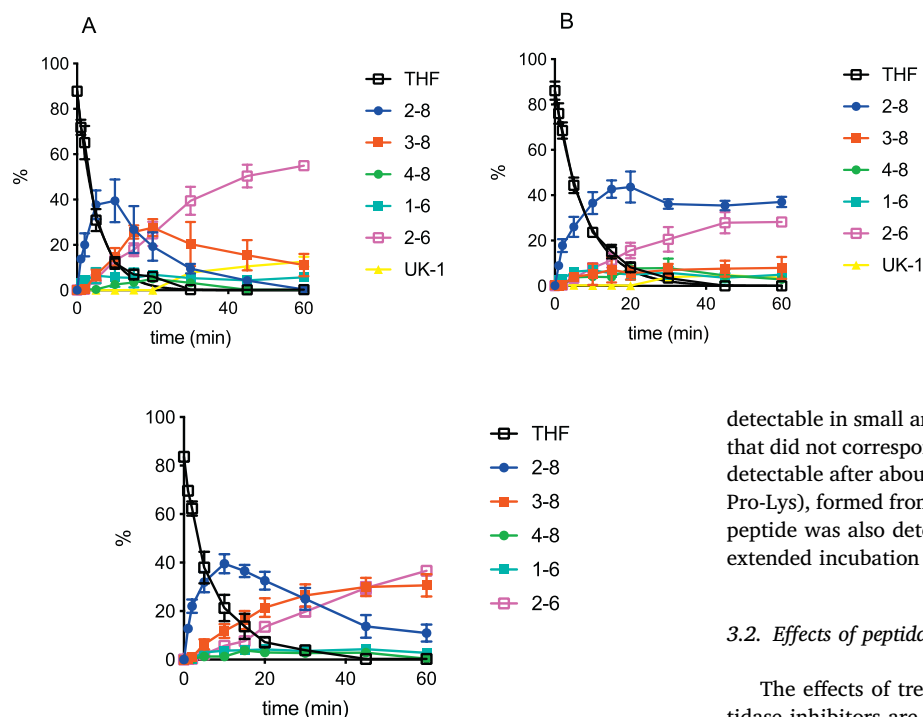
Fig. 2. Time-course of the degradation of THF-γ2 on incubation with human blood. Incubations and TLC analyses were performed as described in the text. Each point is the mean value ± SEM of 3 independent determinations with blood samples from 3 separate individuals.

Comparison of degradation profiles in Figs. 1 and 2 shows the metabolic patterns to be broadly similar for rat and human blood but with some differences when mouse blood was used. Analysis showed that THF was degraded in three main fragments in human and rat blood: the heptapeptide 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu), the hexapeptide 3–8 (Asp-Gly-Pro-Lys-Phe-Leu) and the pentapeptide 2–6 (Glu-Asp-Gly-Pro-Lys).

It can be seen from Figs. 1A and 2 that peptide 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu) appeared rapidly, after only 1 min of incubation in human and rat blood, and that its concentration, expressed as the mean value ± SD % of the total peptides present, increased until about 10 min (39.28% ± 9.3 in rat blood; 39.69% ± 6.9 in human blood). After this time, the concentration of this degradation product started to decrease. Peptide 3–8 (Asp-Gly-Pro-Lys-Phe-Leu) was first detected after about 5 min incubation at 37 °C and was present in good yield after 20 min in rat blood (27.80% ± 3.32) and increased until 60 min in human blood (30.36% ± 7.84). Fragments 1–6 (Leu-Glu-Asp-Gly-Pro-Lys) and 4–8 (Gly-Pro-Lys-Phe-Leu) were detectable in small quantities after extended incubation of THF with human or rat blood. After incubation for 5 min a small amount of 2–6 (Glu-Asp-Gly-Pro-Lys) was detectable (4.72% ± 1.05 in rat blood; 2.16% ± 0.96 in human blood) and its concentration increased until 60 min (54.90% ± 0.87 in rat blood).

Analysis of the behaviour in mouse blood revealed only two major degradation products from [³H]-THF: the heptapeptide 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu) and the pentapeptide 2–6 (Glu-Asp-Gly-Pro-Lys), as shown in Fig. 1B. The peptide 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu) was detectable after 1 min and its concentration increased until 60 min. The peptide 3–8 (Asp-Gly-Pro-Lys-Phe-Leu) appeared as a small peak at 10 min. and remained at approximately the same concentration throughout the remainder of the incubation. The degradation product 2–6 (Glu-Asp-Gly-Pro-Lys) appeared after 5 min and increased until 60 min when it accounted for 36.52% ± 2.69. Other fragments 1–6 (Leu-Glu-Asp-Gly-Pro-Lys) and 4–8 (Gly-Pro-Lys-Phe-Leu) were only

detectable in small amounts (< 10% of total), these included a product that did not correspond to any of the markers used (UK-1). This became detectable after about 20 min and may represent peptide 3–6 (Asp-Gly-Pro-Lys), formed from 2–6, or 4–6 (Gly-Pro-Lys), formed from 4–8. This peptide was also detected in rat (Fig. 1A), but not human, blood after extended incubation periods with THF.

3.2. Effects of peptidase inhibitors

The effects of treatment of the human samples with different peptidase inhibitors are summarized in Fig. 3. Preincubation of the blood samples with either 1,10-phenanthroline (Fig. 3A & B) or amastatin (Fig. 3C & D) produced a marked inhibition of THF degradation (65–86% and 53–75% after incubation of the treated samples with the peptide for 20 min and 60 min, respectively).

In the presence of amastatin the degradation of THF occurred slowly. At concentrations of 1 and 10 μM of amastatin the amounts of fragments 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu) and 2–6 (Glu-Asp-Gly-Pro-Lys) formed were decreased and these were undetectable after treatment with 100 μM or higher concentrations of amastatin. No fragment 3–8 (Asp-Gly-Pro-Lys-Phe-Leu) could be detected at any of the amastatin concentrations used (in the range 1 μM to 1 mM) after either 20 min or 60 min incubation with THF but there was an increase in the concentration of 1–6 (Leu-Glu-Asp-Gly-Pro-Lys) formed.

The degradation patterns observed with the untreated blood samples and summarized in Fig. 4A is consistent with the sequential removal of the amino-terminal amino acids by aminopeptidase activities which were unable to cleave a peptide bond involving proline. Amastatin is an inhibitor of aminopeptidases from several sources [12,13] and thus the above results would support such a conclusion.

The degradation at the C-terminal side of the lysine residue in position 6 would be consistent with the involvement of an enzyme with a trypsin-type specificity. However, this activity was not due to the activity of a such an enzyme since the serine peptidase inhibitor AEBSF (Fig. 3G & H) had little effect on THF degradation. Similar results were obtained with the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) (data not shown).

In the presence of either 10 or 50 mM 1,10-phenanthroline there was only a small quantity of THF degradation (Fig. 3A & B). A much decreased quantity of the fragment 2–8 (Glu-Asp-Gly-Pro-Lys-Phe-Leu) was formed and no fragments 3–8 (Asp-Gly-Pro-Lys-Phe-Leu), 1–6 (Leu-Glu-Asp-Gly-Pro-Lys) and 2–6 (Glu-Asp-Gly-Pro-Lys) were detectable after incubation of the treated blood samples with THF for 20 and 60 min. 1,10-Phenanthroline is an inhibitor of a number of metal-ion containing enzymes. The possibility that the inhibitory action of this compound resulted from binding that was not related to its metal-chelating properties is unlikely, since the alternative chelating agent, EDTA behaved similarly to 1,10-phenanthroline whereas the non-chelating analogue 1,7-phenanthroline was without effect (data not shown). Thus, the above results indicate the involvement of a metallo-aminopeptidases in the sequential degradation from the amino-
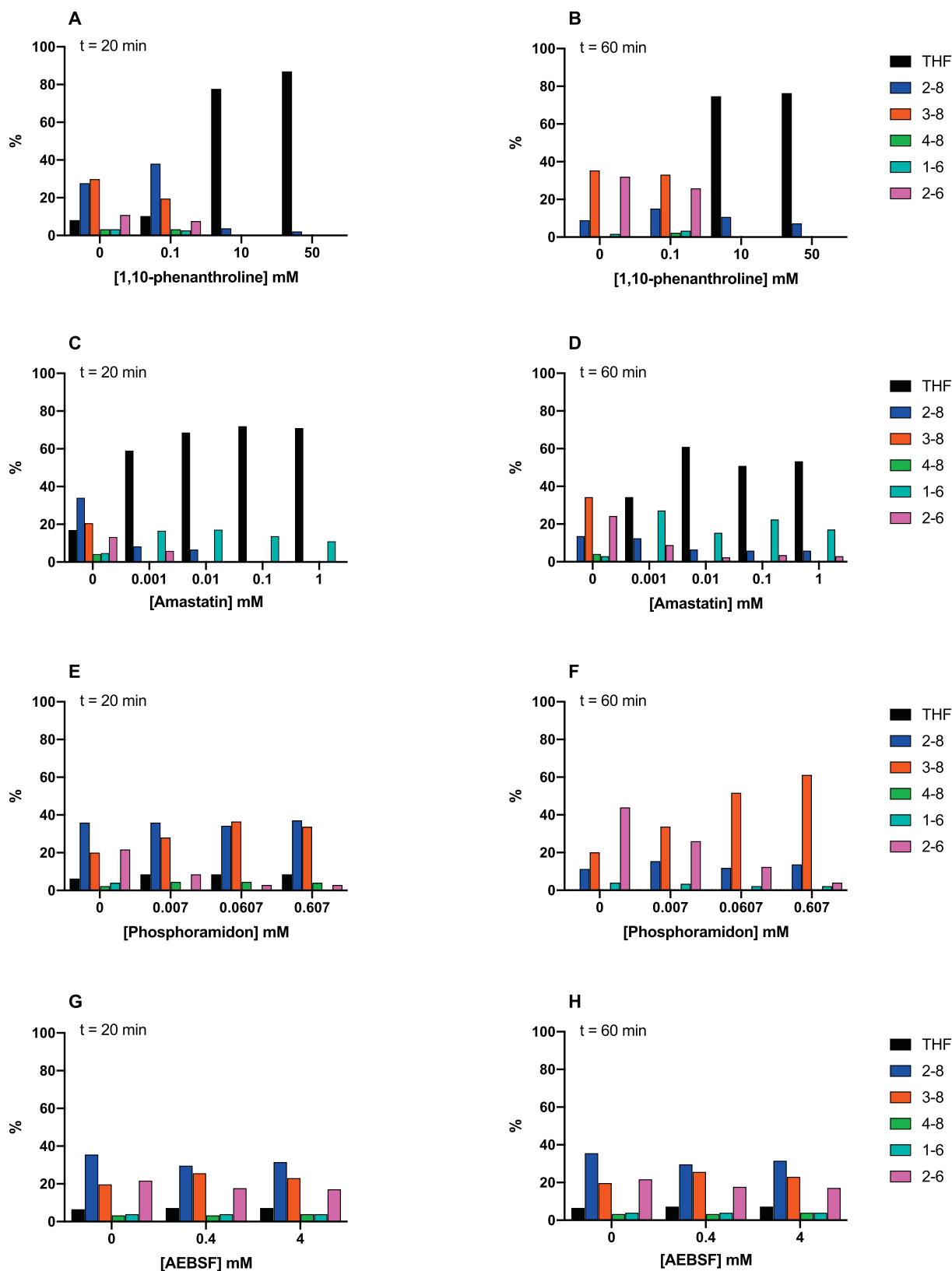
Fig. 3. The effects of peptidase inhibitors on the degradation of THF-γ2 by human blood. The blood samples were pre-incubated with the indicated concentrations of each inhibitor for 30 min at 37 °C before the addition of THF after which incubation was continued for a further 20 or 60 min before the degradation patterns were assessed by TLC. Other details are given in the text. The error bars have been omitted for clarity, but in no case was the range greater than 7% of the actual mean value.
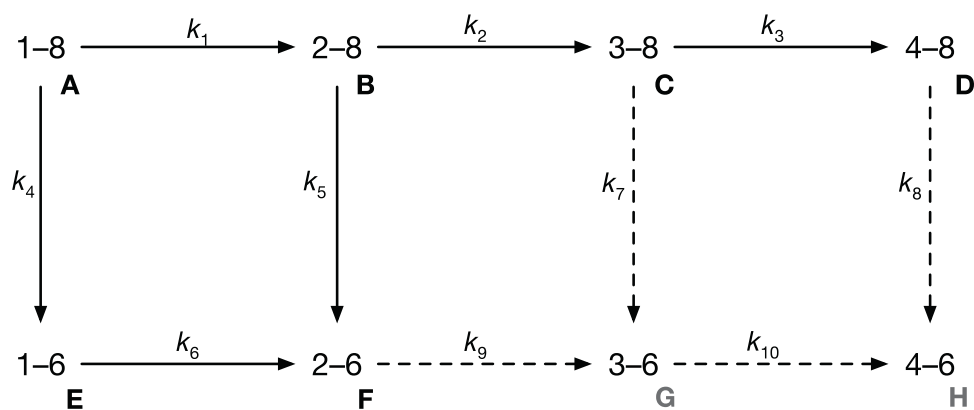
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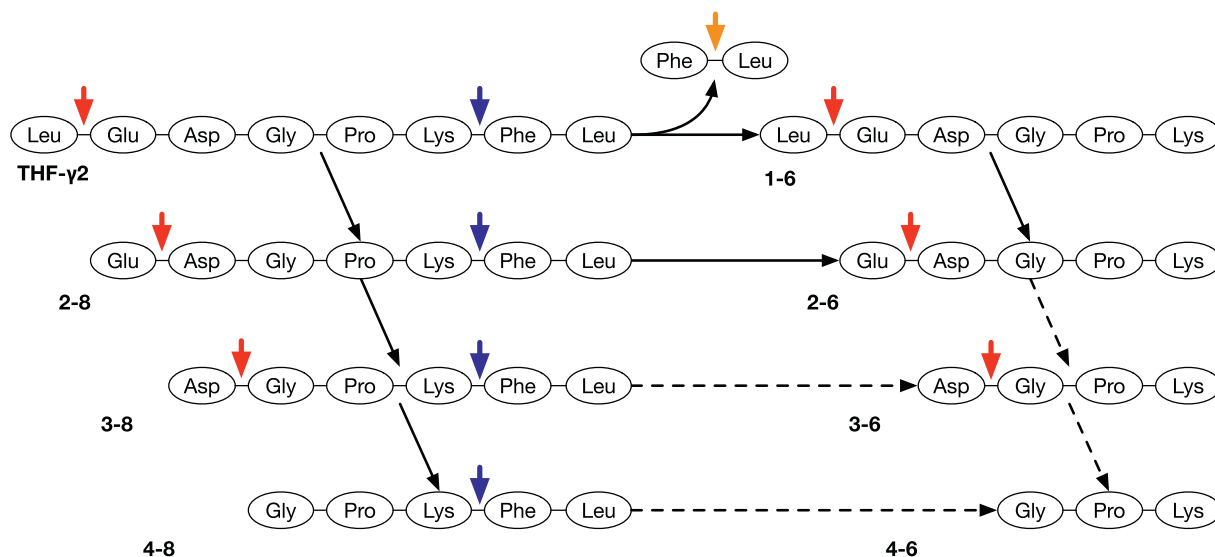
B

Fig. 4. Possible degradative pathways of THF-γ2 on incubation in blood. A) THF and its metabolic byproducts. Enzymes act at the points indicated by the coloured arrows: EC 3.4.11.1, leucyl aminopeptidase (red); EC 3.4.11.7, glutamyl aminopeptidase (blue); EC 3.4.24.27, thermolysin (orange). B) Reaction scheme with first-order rate constants. Solid reaction lines, with rate constants k_1–k_6, are shared by human, rat and mouse, while reactions with dashed lines, k_7–k_{10}, are assumed to be active in the rodent species only. Variables of the model *A–F* are metabolites present in all species, while *G* and *H* are potential products in rat and mouse.

terminus and a metallo-endopeptidases in the cleavage of the Lys-Phe bond at position 6–7.

Phosphoramidon is an inhibitor of some metallo-endopeptidases such as membrane metallo-endopeptidases (EC 3.4.24.11) [14], and the thermolysin (EC 3.4.24.27) group [15]. However these enzymes are also inhibited by 1,10-phenanthroline. In the present case, as shown in Fig. 3E & F, phosporamidon at concentrations ranging between 7 and 607 μM appeared to be without significant effect on the aminopeptidase activity, but the cleavage of the Lys-Phe bond (position 6–7 in the peptide) was inhibited resulting in an increase in the concentration of the fragment 3–8 (Asp-Gly-Pro-Lys-Phe-Leu) and decreases of 2–6 (Glu-Asp-Gly-Pro-Lys) and 1–6 (Leu-Glu-Asp-Gly-Pro-Lys).

3.3. Modelling of the degradation patterns

The overall catabolic pattern for THF is summarized in Fig. 4A. The reactions can be modelled according to the scheme shown in Fig. 4B, in

which it is assumed, for simplicity, that each of the degradative steps can be represented by a first-order process and the relative concentrations of each of the products formed will be governed by the concentrations of their precursors and the relative values of the rate constants for their formation and degradation. The first-order assumption is justified in view of the relatively high K_m values of the peptidases involved and the low concentration of THF used, in which case the apparent constants will represent V_{max}/K_m, or $k_{cat}[E]_0/K_m$, for each enzyme. Although the further degradation products 3–6 and 4–6 may be formed, they were not detected in significant quantities in human blood within the time-course of the experiments and thus their rates of formation may be neglected for the purposes of this analysis.

The rate equations for each of these linked processes are shown in Eqs. 1.

$$\frac{dA}{dt} = -(k_1 + k_4)A$$

$$\frac{dB}{dt} = k_1 A - (k_2 + k_5)B$$

$$\frac{dC}{dt} = k_2 B - k_3 C$$

$$\frac{dD}{dt} = k_3 C$$

$$\frac{dE}{dt} = k_4 A - k_6 E$$

$$\frac{dF}{dt} = k_6 E + k_5 B \tag{1}$$

The Laplace Transform Method is a convenient and robust means for obtaining solutions to networks of competing first-order ordinary differential equations (ODEs) through algebraic manipulation [10,16]. Assuming that the initial concentrations of all peptide metabolites are zero, and denoting the initial concentration of THF by A_0, the Laplace Transform Method yields expressions for the concentrations of the metabolites shown in Eqs. 2–7, in which the following additional definitions have been used: $k_a = k_1 + k_4$, $k_b = k_2 + k_5$, $k_c = k_3$, $k_e = k_6$, $B_0 = k_1 A_0$, $C_0 = k_1 k_2 A_0$, $D_0 = k_1 k_2 k_3 A_0$, $E_0 = k_4 A_0$ and $F_0 = k_1 k_5 A_0$.

$$A(t) = A_0 e^{-k_a t} \tag{2}$$

$$B(t) = \frac{B_0}{k_b - k_a}(e^{-k_a t} - e^{-k_b t}) \tag{3}$$

$$C(t) = C_0\left(\frac{e^{-k_a t}}{(k_b - k_a)(k_c - k_a)} + \frac{e^{-k_c t}}{(k_a - k_c)(k_b - k_c)} + \frac{e^{-k_b t}}{(k_a - k_b)(k_c - k_b)}\right) \tag{4}$$

$$D(t) = \frac{D_0}{k_a k_b}\left(1 - \frac{k_b k_c}{(k_a - k_b)(k_a - k_c)}e^{-k_a t} + \frac{k_a k_c}{(k_a - k_b)(k_b - k_c)}e^{-k_b t} - \frac{k_a k_b}{(k_a - k_c)(k_b - k_c)}e^{-k_c t}\right) \tag{5}$$

$$E(t) = \frac{E_0}{k_a - k_e}(e^{-k_e t} - e^{-k_a t}) \tag{6}$$

$$F(t) = \frac{F_0 + E_0 k_b}{k_a k_b} + \frac{F_0(k_a - k_e) + E_0 k_e(k_a - k_b)}{k_a(k_a - k_b)(k_a - k_e)}e^{-k_a t} - \frac{F_0}{k_b(k_a - k_b)}e^{-k_b t} - \frac{E_0}{(k_a - k_e)}e^{-k_e t} \tag{7}$$

A graph of these functions as applied to the metabolism of THF in human blood is shown in Fig. 5. Apparent first-order rate constants
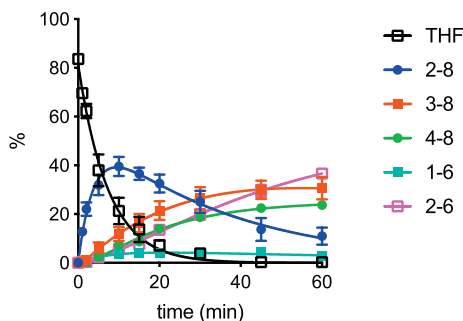

Fig. 5. Analytical solutions obtained by the Laplace Transform Method (Eqs. 2–7) fitted by non-linear regression to the data from human blood (Fig. 2). Data points represent mean values ± SEM of 3 independent determinations with blood samples from 3 separate individuals.

were derived from the concentration-time curves for each substrate by non-linear regression in Prism. The half-life of THF in human blood can be calculated from the relationship $t_{1/2} = (\ln 2)/k_a = 5.2 \pm 0.4$ min. A similar calculation, for the mouse data of Fig. 1b, gave a half-life of 5.6 ± 0.2 min. Degradation of THF in rat blood (half-life 3.7 ± 0.2 min) was somewhat faster than either mouse (p < 0.0001) or human (p < 0.001) blood.

While analytical solutions to systems of linear ODEs with constant coefficients can be obtained comparatively easily using the method of Laplace transforms, the use of curve-fitting methods to estimate the parameters is hampered by the high degree of sensitivity of higher-order exponential functions to measurement error [17]. Furthermore, the slow rate of formation of some of the products and the low levels obtained within the time of the experiments will compound the curve-fitting errors. Nevertheless, the satisfactory agreement between the simulated curve in Fig. 5 and the experimentally observed results suggests that the model shown in Fig. 4B provides a good explanation for the degradation process.

In Fig. 1A, the majority of the flux appears to end in 2–6, with 1–6 appearing only briefly as an intermediate. UK-1 starts to increase as 3–8 peaks, thereafter their traces appearing to mirror the other, which might indicate a reaction of 3–8 to 3–6, according to the scheme of Fig. 4B. Similarly, 4–8, which appears as the final degradation product in human blood, in rat and mouse, appears in low amounts, peaking at 20–30 min, suggesting 4–6 as a possible candidate for the unknown compound in rat. The model for the degradation of THF in human blood was therefore adapted to include additional rate constants for the formation of 3–6 (Asp-Gly-Pro-Lys), formed from 2 to 6, and 4–6 (Gly-Pro-Lys), formed from 3 to 6 or 4–8. The ODE system (1) was modified to include additional equations representing the formation of 3–6 (G) and 4–6 (H),

$$\frac{dG}{dt} = k_7 C - k_{10} G$$

$$\frac{dH}{dt} = k_8 D + k_{10} G$$

with dC/dt modified to include the additional term $-k_7 C$ to account for the formation of 3–6 from 3 to 8 (Fig. 4B). The new system of ODEs was integrated numerically in R [18] using the deSolve library. Fig. 6 shows the simulated time courses of the rat model, which reproduce aspects of both the rat and mouse data (Fig. 1), including the delayed rise of the "unknown" peptide, here taken to be 4–6. A lower rate of decay of THF was chosen, according to the findings in mouse. In the absence of inhibition, the percentage of 3–6 would be expected to rise to a proportion of the initial THF percentage, with the remainder being the terminal product, 4–6. The decline in activity after 30 min, which is evident in the mouse data, could be an indication of enzyme inactivation, or the presence of an inhibitor, neither of which are accommodated by the present linear model.

4. Discussion

As might be expected from the peptidases present in whole blood, the degradation of THF in human blood showed a more diverse pattern of products than that reported for plasma by Bramucci et al. [7]. The differences are summarized in Fig. 7. Only three major products with Leu-Glu-Asp-Gly-Pro-Lys (1–6) being formed rapidly and Glu-Asp-Gly-Pro-Lys (2–6) and Phe-Leu (7–8) appearing more slowly in plasma. The half-life of THF in plasma was calculated to be about 12 min.

Comparison with the behaviour in whole blood, reported here, shows a more rapid degradation with a half-life of less than 4 min, which is over three times lower than the value obtained in plasma [7]. Metabolism involved the rapid transient formation of Glu-Asp-Gly-Pro-Lys-Phe-Leu (2–8) followed by a slower formation of Asp-Gly-Pro-Lys-Phe-Leu (3–8) and 2–6 with small increases in 4–8 and 1–6. The Gly-Pro bond was not hydrolysed in either preparation. The inhibition by 1,10-
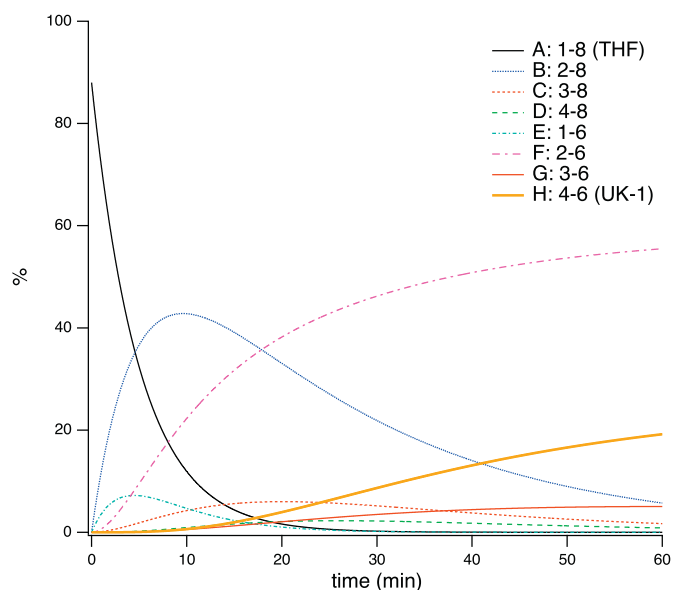
Fig. 6. Modelling the degradative pathways of THF on incubation with rat blood. The values used for the first-order rate constants, in min^{-1}, for the individual steps shown in Fig. 4B, were calculated, as described in the text, as $k_1 = 0.15$, $k_2 = 0.02$, $k_3 = 0.08$, $k_4 = 0.05$, $k_5 = 0.025$, $k_6 = 0.25$, $k_7 = 0.03$, $k_8 = 0.2$, $k_9 = 0.0$, $k_{10} = 0.01$. Additional parameters used: $A_0 = 88.0$.
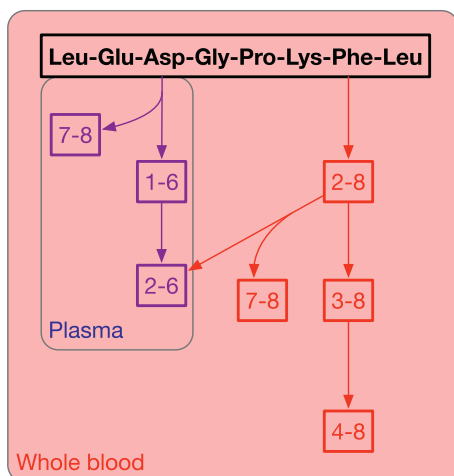

Fig. 7. Comparison of the THF-γ2 metabolic fragments determined in human blood with those reported for human blood plasma [7].

phenthroline indicates the involvement metallo *exo-* and *endo-*peptidases in the degradation from the amino-terminus and the hydrolysis of the Lys-Phe bond, respectively. Clearly, a leucyl-aminopeptidase (EC 3.4.11.1) activity is present in both systems leading to the formation of 2–8. That enzyme is known to be inhibited by amastatin and chelating agents. The formation of the further N-terminal degradation products 3–8 and 4–8 is consistent with the activity of glutamyl aminopeptidase (EC 3.4.11.7), an enzyme that has been shown to be present in blood serum. Although this zinc-containing enzyme is sensitive to inhibition by 1,10-phenthroline and amastatin, the inhibitor studies would not be expected to reveal this since removal of the terminal leucine would be necessary for the enzyme to act. Cleavage of the Lys-Phe bond to produce 1–6 and 2–6 was catalysed by a phosphoramidon-sensitive metallo-endopeptidase. These enzymes belong to the M13 (neprilysin) family of peptidases, as classified by the MEROPS peptidase database [19], which includes, in addition to neprilysin (neutral endopeptidase; EC 3.4.24.11) [20,21], peptidyl-dipeptidase A (ACE, angiotensin

converting enzyme; EC 3.4.15.1) [22] and the erythrocyte Kell protein [23]. Both peptidyl-dipeptidase A [7] and neprilysin [21] have been shown to catalyse this cleavage in isolated THF. This activity can be seen to have a proportionally greater role in the degradation in plasma than it does in whole blood. The presence of heparin, which has been shown to inhibit neprilysin [24], may have affected the results, although that anticoagulant was present in both the plasma and whole-blood samples.

Studies with synthetic analogues of THF have shown the Phe residue at position 7 to be important to its effects on the impaired blastogenic response of phytohemagglutinin(PHA)-stimulated T-lymphocytes from uremic patients with infectious diseases [25,26]. The degradation patterns in each of the species indicate that the facility in which this is removed by cleavage of the Lys-Phe bond, to yield peptides 1–6 and 2–6, may be an important factor in limiting the effectiveness of THF-γ2 in vivo. However, amino-acid substitutions at positions 1–5 have also suggested that alterations to each of these residues decrease the potency of THF-γ2 indicating that proteolysis at the N-terminal will also be deleterious. From this perspective, the differences in degradation patterns between mouse, rat and human may be of little importance. However, mouse would appear to be a less satisfactory model for the proteolysis in blood than the rat, although further work would be necessary to determine whether this behaviour was specific to the mouse strain used or to their age or weight. As might be expected the variation between the three human samples was greater than those from the rodents, but this affected the rates of product formation rather than the nature of the products formed. It would be of interest in the future to investigate the effects of diseases, such as metabolic syndrome and type 2 diabetes, where the plasma levels of neprilysin and peptidyl-dipeptidase A have been shown to be elevated [27,28]. Inhibitors of both these enzymes, which have been used in hypertension and have been suggested to be useful the treatment of type 2 diabetes [29,30], may also prolong the effectiveness of THF-γ2. The suggestion that ACE inhibitors may be of value in the treatment of coronavirus COVID-19 raises the possibility that the addition of THF-γ2 might be beneficial because of its immune-stimulating actions [31].

The model of THF degradation pathways by numerical solution of the differential equations has been shown to approximate the data from mouse and rat, although further work is needed to confirm the identity of the unknown products. For the human samples, in which all detected peptides were identifiable, the method of Laplace transforms permitted an analytical solution to the model, from which estimates of the fitting parameters could be obtained by nonlinear regression. This analytical method could be applied to the degradation of other peptides, and other deterministic systems, where the substrate concentrations are sufficiently lower than the K_m values of the enzymes involved that apparent first-order kinetics are followed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

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