VALPROYL-DEPHOSPHOCoA: A NOVEL METABOLITE OF VALPROATE FORMED IN VITRO IN RAT LIVER MITOCHONDRIA

Margarida F. B. Silva, Lodewijk IJlst, Paul Allers, Cornelis Jakobs, Marinus Duran, Isabel Tavares de Almeida, and Ronald J. A. Wanders

Department of Clinical Chemistry and Pediatrics, University of Amsterdam, Amsterdam, The Netherlands (M.F.B.S., L.I., M.D., R.J.A.W.); Centro de Patogénesis Molecular, Faculdade de Farmácia da Universidade de Lisboa, Lisboa, Portugal (M.F.B.S., I.T.d.A.); University Children’s Hospital, Utrecht, The Netherlands (P.A.); and Department of Clinical Chemistry, Free University Hospital, Amsterdam, The Netherlands (C.J.)

Received October 10, 2003; accepted July 22, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
The mitochondrial metabolism of valproic acid (VPA) was investigated in vitro to elucidate its β-oxidation pathway since the characterization of VPA intermediates in the acyl-CoA thioester form, and not just in their free acid form, has not been fully achieved. Intact rat liver mitochondria were incubated with [4,5-3H2]VPA and [2-3H]VPA. The respective intermediates, valproyl-CoA, Δ2β,β-valproyl-CoA, 3-hydroxyvalproyl-CoA, and 3-oxovalproyl-CoA were analyzed by reverse phase high performance liquid chromatography (HPLC) with radioisotope and UV detection. An unknown metabolite, originating from both labeled substrates, was detected. It was identified as valproyl-dephosphoCoA (valproyl-dephCoA) by fast atom bombardment mass spectrometry (FAB-MS) analysis of the corresponding HPLC peak fraction. The FAB-MS spectrum of the authentic chemically synthesized valproyl-dephCoA proved to be consistent with that of the unknown compound. Valproyl-dephCoA is produced from valproyl-CoA in mitochondria, probably via a phosphatase-catalyzed reaction. This conversion was shown to be more dependent on the energy state involving [AXP] ([AXP] = [ATP] + [ADP] + [AMP]) and [phosphate] concentrations rather than the strict mitochondrial [ATP]/[ADP] ratio. The results indicate that higher concentrations of AXP and phosphate inhibit the dephosphorylation of valproyl-CoA. A complete understanding of the toxic significance of valproyl-dephCoA formation in vivo as a potential inhibitor of fatty acid β-oxidation is important to clarify the pathogenesis of VPA-associated hepatotoxicity.

The metabolism of the antiepileptic drug valproic acid (VPA) involves both phase I (oxidative) and phase II (conjugative) biotransformation pathways. Despite the fact that VPA is a simple eight-carbon branched-chain fatty acid, VPA metabolism is extremely complex, involving more than 50 known metabolites (Abbott and Anari, 1999). The progress of sensitive and specific analytical methods such as gas chromatography-mass spectrometry (Nau et al., 1981; Tatsuhara et al., 1987; Rettenmeier et al., 1989) or liquid chromatography-mass spectrometry (Kassahun et al., 1994; Tang and Abbott, 1996) has greatly improved the identification of numerous metabolites, including stereo- and geometric isomers, found predominantly in their free forms but also as conjugates (Gopaul et al., 2000, 2003).

Mitochondrial β-oxidation of VPA is the predominant oxidative process in humans, whereas cytochrome P450-mediated metabolism, including (ω)- and (ω-1)-oxidation, represents minor phase I processes of VPA metabolism (Granneman et al., 1984; Davis et al., 1994). The characterization of the complex enzymatic processes involved in VPA β-oxidation has not been fully achieved. Once inside the mitochondria, VPA will generate four intermediate acyl-CoA esters by the four concerted reactions of the β-oxidation cycle (Li et al., 1991; Silva et al., 2001). Currently, available evidence suggests that several individual enzymes involved in this pathway are different from those required for the β-oxidation of straight-chain fatty acids (Ito et al., 1990; Li et al., 1991; Silva et al., 2002). The elucidation of VPA biotransformation in mitochondria, including the detection of new cellular metabolites, is crucial to understanding the mechanism of its hepatotoxicity, which remains obscure (Stephens et al., 1992; König et al., 1994, 1999; Graf et al., 1998; Radatz and Nau, 1999).

The main goal of the present investigation was to study the fate of VPA in mitochondria by monitoring its intermediates, valproyl-CoA, Δ2β,β-valproyl-CoA, 3-hydroxyvalproyl-CoA, and 3-oxovalproyl-

ABBREVIATIONS: VPA, 2-n-propylpentanoic acid or valproic acid; Δ2β,β-VPA, 2-n-propyl-trans-2-pentenoic acid; CoA, coenzyme A; dephCoA, dephosphocoenzyme A; P, orthophosphate; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; ATP-γ-S, adenosine-5′-O-(3-thiotriphosphate); AXP, total adenine nucleotides ([AMP] + [ADP] + [ATP]); MOPS, 3-[N-morpholino]propanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′′-tetraacetic acid; SEM buffer, sucrose/EGTA/MOPS buffer; KP, buffer, potassium dihydrogen phosphate buffer; FAB-MS, fast atom bombardment mass spectrometry; HPLIC, high performance liquid chromatography; radio-HPLC, high performance liquid chromatography with radiochemical detection; RLM, rat liver mitochondria.
CoA using reverse phase radio-high performance liquid chromatography (radio-HPLC) analysis. During this study, an unknown metabolite, generated from the metabolism of valproate in mitochondria, was detected. It was identified as valproyl-dephosphoCoA, and the hypothesis that it was primarily formed by dephosphorylation of valproyl-CoA was further investigated.

Materials and Methods

Materials. VPA, bovine serum albumin (fatty acid-free), 3'-dephosphoacetyl-CoA, adenosine 5'-monophosphate (AMP, sodium salt), bicinechonic acid, and other standard biochemicals were obtained from Sigma-Aldrich (St. Louis, MO). Δ²(E)-VPA was a kind gift of Prof. Dr. D. Lindhout (Erasmus University of Rotterdam, The Netherlands). Coenzyme A (CoA; grade II, trilithium salt), adenosine 5'-diphosphate (ADP; sodium salt), adenosine 5'-triphosphate (ATP; sodium salt), and adenosine 5'-O-(3-thiotriphosphosphate) (ATP-γ-S; tetralithium salt) were supplied by Roche Diagnostics (Mannheim, Germany). Lichrosolv-grade solvents for high performance liquid chromatography (HPLC) were obtained from Merck (Darmstadt, Germany). [4,5-3H₂]Valproic acid (specific activity = 50 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA) and [2-3H]valproic acid, sodium salt (specific activity = 15.0 Ci/mmol), was acquired from American Radiolabeled Chemicals. (St. Louis, MO). Ultima Flo AP scintillation cocktail was purchased from PerkinElmer Life and Analytical Sciences (Gröningen, The Netherlands).

Preparation of Mitochondria from Rat Liver. Adult male Wistar rats (about 250 g) were starved for 18 h and sacrificed by decapitation. Subsequently, the livers were removed and immediately rinsed into ice-cold homogenization medium (25–50 mg/ml). The livers were removed and immediately rinsed into ice-cold homogenization medium (25–50 mg/ml). The rat liver mitochondria (RLM) suspension was frozen at −70°C for further use as mitochondrial homogenate.

Synthesis of Valproyl-CoA, Δ²(E)-Valproyl-CoA, 3-Hydroxyvalproyl-CoA, and Valproyl-dephosphoCoA. The CoA thioesters of VPA, Δ²(E)-VPA, and 3-hydroxy-VPA were prepared by a mixed anhydride method using ethylchloroformate, adapted from the method of Rasmussen et al. (1990). Δ²(E)-Valproyl-CoA was used as the precursor for the synthesis of 3-oxovalproyl-CoA using sulfolipid RLM as a source of enzyme. The detailed procedures for the synthesis, identification, purification, and quantification of valproyl-CoA, Δ²(E)-valproyl-CoA, 3-hydroxyvalproyl-CoA, and 3-oxovalproyl-CoA have been published elsewhere (Silva et al., 2001). Valproyl-dephosphoCoA was synthesized as described for valproyl-CoA (Silva et al., 2001), using VPA and dephosphoCoA as precursors. Valproyl-dephosphoCoA was further purified by solid-phase extraction and by preparative HPLC, and its identity was confirmed by fast atom bombardment mass spectrometry (FAB-MS).

Mitochondrial Incubations with [3H]Valproic Acid in Intact RLM. Incubations were performed at 37°C in a final volume of 250 µl containing freshly isolated mitochondria (5 mg/ml) suspended in the basal isotonic isolation buffer at pH 7.4 (250 mM sucrose, 2 mM EGTA, 5 mM NaMOPS (SEM) in a shaking water bath. Incubations for the analysis of intermediates were started by addition of 20 pmol of [4,5-3H₂]VPA or 60 pmol of [2-3H]VPA, after previous evaporation of the ethanol present in the stock solution, and redisubmitted in the incubation buffer.

Studies were done with and without the simultaneous inclusion of nonlabeled VPA (0.1–2.0 mM). Reactions were stopped at appropriate times (0.25, 0.5, 1, 3, 6, 10, 30, 60, and 120 min) by addition of 25 µl of 2 M HCl in a vortex mixer. Protein was removed by centrifugation (17,000 × g /min/4°C) in an Eppendorf centrifuge, and the supernatant was neutralized with 25 µl of 2 M KOH/0.6 M 2-[N-morpholino]ethanesulfonic acid during mixing in a vortex mixer. After centrifugation (17,000 × g /min/4°C), the supernatant was transferred to microvials to be analyzed by radio-HPLC. RLM were also incubated using both tritiated VPA substrates, with and without nonlabeled VPA, in the presence of 4 mM MgCl₂, bovine serum albumin (0.5 mg/ml), 0.5 mM malate, 1 mM adenosine-5'-diphosphate (ADP), and 4 mM adenosine-5'-triphosphate (ATP).

Alkaline hydrolysis of the mitochondrial incubations was performed by adding the medium (250 µl) to 100 µl of 2 M NaOH and by heating at 50°C for 30 min. After this period, samples were acidified with 100 µl of 2 M HCl and then neutralized with 1 M acetate buffer, pH 6. Protein was removed by centrifugation at 17,000 × g for 5 min at 4°C, and the supernatant was analyzed by radio-HPLC.

Radio-HPLC Analysis of VPA Acyl-CoA Esters. A Waters model 510 HPLC pump (Waters, Milford, MA) was used as the solvent delivery system, with a Waters gradient controller model 600, and the column was a Supelcosil LC-18-S (5-µm). The UV detector was at 210 nm. The mass spectrometry detection was performed using a JEOL JMS-AX505SW mass spectrometer (JEOL, Tokyo, Japan).
range was set at 200 to 1500 m/z. The dephCoA-ester was dissolved in a matrix consisting of glycerol/methanol/H₂O (50:35:15, v/v). A 3-µl aliquot was applied to the FAB probe.

Mitochondrial Incubations with Valproyl-CoA in RLM Homogenate. Freshly isolated mitochondria kept at −70°C were thawed in ice-cold water, diluted with PBS, and subjected to mild sonication (twice for 10 s, 80 W, 4°C) to ensure complete lysis of the organelles. Incubations were done in a shaking water bath at 37°C in a final volume of 250 µl. Reactions were started by adding mitochondrial lysate (5 mg/ml) to a mixture, preincubated for 5 min at 37°C, containing the isolation (SEM) buffer, potassium dihydrogen phosphate (KP) buffer (0–50 mM), valproyl-CoA (0–1 mM), 0.5 mM MgCl₂, ATP, ADP, AMP [total adenine nucleotides (AXP) = 0–15 mM]. Incubations were allowed to proceed for different periods (0–4 h). Reactions were terminated by addition of 25 µl of 2 M HCl. Precipitated protein was removed by centrifugation, and the supernatant was neutralized with 2 M KOH/0.6 M 2-[N-morpholino]ethanesulfonic acid. After centrifugation (17,000 g/5 min/4°C), the neutralized supernatant was analyzed by HPLC with UV detection.

For a total [AXP] concentration of 2.5 mM, 5.0 mM, 10 mM, and 15 mM, the individual concentrations of ATP, ADP, and AMP were calculated in each assay (see Fig. 7), considering the reversibility of their conversion catalyzed by adenylate kinase, which was assumed to be at equilibrium (ATP + AMP = 2ADP; Kₐₑₒ = 1). Furthermore, as initial experimental condition, the ratio [ATP]/[ADP] was established as 4 or 0.5, as described for respiratory state 4 and state 3, respectively. The calculated initial energy charge, defined as the effective molar fraction of ATP: (ATP + 0.5×ADP)/(ATP + ADP + AMP) (Atkinson, 1977; Zubay, 1998), corresponds to 0.86 and 0.29 in the present study.

Mitochondrial Incubations with Valproyl-dephosphoCoA. For this purpose, valproyl-dephCoA was used as precursor, and the formation of valproyl-CoA was followed by HPLC analysis. Reactions were started by adding mitochondrial lysate (5 mg/ml) to a mixture preincubated for 5 min at 37°C, containing the isolation (SEM) buffer, valproyl-dephCoA (0–1 mM), (±) 0.5 mM MgCl₂, and ATP (0–5 mM). Incubations were allowed to proceed for different periods (0–4 h) at 37°C. Reactions were terminated as described above, as well as the HPLC analysis (UV detection) of the supernatants.
Data Analysis. The present procedure for the HPLC determination of valproyl-CoA or valproyl-dephospho-CoA in mitochondrial homogenates was validated by assessing the intraassay reproducibility and within-day variability by quantification of both compounds in four aliquots of the same sample [results: 6.2% (n = 4) for the former, and 3.5% (n = 4) for the latter]. Interassay reproducibility or day-to-day variability was assessed by quantifying the same standards analyzed on different days [results: 7.9% (n = 15) for valproyl-CoA and 9.2% (n = 14) for valproyl-dephospho-CoA].

Each value shown in Figs. 4 to 7 represents the mean (±S.D.) of two to four independent assays corresponding to different mitochondrial preparations, incubations, or HPLC injections. The estimated value for the standard deviation for each point was less than 10%, and it is indicated in the figures as a corresponding error bar. Due to the limited availability of the standards of valproyl-CoA and valproyl-dephospho-CoA, which were previously synthesized and purified, not all the determinations in the referred figures were done in quadruplicate. Where possible (n = 4), mean values were compared by Student’s t test (unpaired, two-tailed, unequal variance), and a statistical significance was defined as p ≤ 0.05.

Results

HPLC and Radio-HPLC Analysis of VPA Metabolites. The activation and metabolism of VPA in RLM were monitored by radio-HPLC. Figure 2A depicts the chromatogram obtained by radio detection of the valproyl-CoA metabolites generated from [4,5-3H2]VPA in RLM for 10 min. Besides the four indicated acyl-CoA esters, formed by the four sequential reactions of the β-oxidation cycle, a more hydrophobic peak was also detected (peak 6). This unknown product was also UV-absorbing (258 nm), as shown in Fig. 2C. Alkaline hydrolysis of the sample caused the disappearance of this peak together with the acyl-CoA esters. To elucidate the chemical nature of this metabolite, the same experimental conditions were reproduced,
but using a different substrate, [2-3H]VPA. The respective radio- and UV-chromatograms revealed that the unknown product is also formed following the reaction catalyzed by having a molecular mass of 893, gave a base peak at 813, as deduced from the base peak of the mass spectrum at \( m/z = 812 ([M - H]^+) \). Valproyl-CoA, having a molecular mass of 893, gave a base peak at \( m/z = 892 \) (data not shown). The apparent loss of 80 mass units in the unknown substance is compatible with the loss of a phosphate group. In addition, it was demonstrated that the chemically synthesized valproyl-dephosphoCoA presented liquid chromatographic properties (HPLC) (Fig. 2, E and F) identical to, and a FAB-MS spectrum consistent with, those of the unknown metabolite (Fig. 3B).

**Identification of Valproyl-dephosphoCoA.** The FAB mass spectrum of the unknown metabolite, identified as VPA-dephosphoCoA, is shown in Fig. 3A. All mass spectrometric investigations were carried out with pooled fractions, which had been collected from several incubations with nonlabeled VPA. The molecular mass of the obtained product appeared to be 813, as deduced from the base peak of the mass spectrum at \( m/z = 812 ([M - H]^+) \). Valproyl-CoA, having a molecular mass of 893, gave a base peak at \( m/z = 892 \) (data not shown). The apparent loss of 80 mass units in the unknown substance is compatible with the loss of a phosphate group. In addition, it was demonstrated that the chemically synthesized valproyl-dephosphoCoA presented liquid chromatographic properties (HPLC) (Fig. 2, E and F) identical to, and a FAB-MS spectrum consistent with, those of the unknown metabolite (Fig. 3B).

**Formation of Valproyl-dephosphoCoA in Intact RLM.** The time course of VPA metabolism in intact RLM was followed by monitoring the production of the various VPA metabolites, during 4 h. The disappearance of [3H]VPA and the respective formation of valproyl-CoA and/or valproyl-dephospho-CoA were virtually linear up to an incubation period of 10 min. The maximum concentration of valproyl-dephosphoCoA was reached within 6 to 10 min, followed by a progressive disappearance (up to 2 h). Its intramitochondrial concentration was calculated both in state 3 (addition of ADP) and state 4 (resting state), assuming an intramitochondrial water space of 1.0 \( \mu l/mg \) of protein (LaNoue et al., 1973). Using an incubation time of 10 min, a protein concentration of 5 mg/ml, and the precursor (VPA) concentration of 0.1 mM, the following quantitative results were obtained: \([\text{valproyl-dephCoA}] = 162 \pm 30 \mu M \) (state 4; \( n = 5 \)) and \( 27 \pm 9 \mu M \) (state 3; \( n = 3 \) (mean \pm S.E.M.). This quantification was based on the calibration curve obtained with the standard valproyl-CoA, and an equal response factor was assumed for valproyl-dephCoA.

**Formation of Valproyl-dephosphoCoA in RLM Homogenate.** When a pure solution of valproyl-CoA was incubated up to 2 h in SEM buffer at 37°C in the absence of a mitochondrial extract, no valproyl-dephCoA was produced. However, upon the incubation of valproyl-CoA in SEM buffer at 37°C in the presence of a mitochondrial homogenate, valproyl-dephCoA was formed (Fig. 3).

As shown in Fig. 4A, a linear rate of disappearance of valproyl-CoA was observed within the first 10 min of incubation, which was inversely related to the amount of ATP (1.37 versus 0.63 nmol/min/mg protein for [ATP] = 0 and 1 mM, respectively). Figure 4B shows that valproyl-dephCoA was formed at approximately the same rate as the disappearance rate of valproyl-CoA. An incubation period of 10 min was chosen for the next experiments. After this period, the sum of both peak areas (remaining valproyl-CoA and formed valproyl-dephCoA) was about 80% of the initial valproyl-CoA area (\( \Delta t = 0 \)), suggesting that only 20% of the substrate was consumed by \( \beta \)-oxidation and/or hydrolysis and not converted to valproyl-dephCoA. To study valproyl-dephCoA formation in more detail, we investigated the effect of the substrate (valproyl-CoA), AXP, and KPi concentrations. After 10 min, the sum of both peak areas (remaining substrate and formed product) correlated linearly with the increase of valproyl-CoA (Fig. 5A). This correlation stresses the constant contribution of other enzymatic reactions besides dephosphorylation during this period. As shown in Fig. 5B, the increase of the precursor concentration leads to an increase of its dephosphorylation, whether ATP is present or not. However, the formation rate of valproyl-dephCoA is clearly higher in the absence of ATP, with a greater increment for lower concentrations of substrate. The study of the effect of the ATP concentration in the medium is depicted in Fig. 6A. The formation rate of valproyl-dephCoA from valproyl-CoA (0.1 mM) was found to decrease to a constant level for [ATP] \( \geq 0.75 \) mM. A similar inhibition was obtained when ATP was replaced by ATP-\( \gamma \)-S, a nonhydrolyzable ATP analog used in the same concentration range. The observed decrease was linear, however, and not exponential as observed with ATP. The KPi concentration was also found to be highly determinant on the amount of valproyl-dephCoA formed from a constant amount of precursor. The results are shown in Fig. 6B, where for [KPi] \( \geq 5 \) mM, the obtained rate is less than 30% of the one obtained in the absence of exogenous KPi.

To investigate how the ATP-ADP-AMP pool would affect the rate and extent of valproyl-dephCoA formation, two extreme conditions such as a high ATP/ADP ratio (high energy charge as in respiratory state 4) and a low ATP/ADP ratio (low energy charge as in respiratory state 3) were applied. These independent factors were studied in the absence of added KPi, and in its presence (20 mM). The overall results are presented in Fig. 7 showing that, apparently, no clear differences exist between these two conditions (graph A comparable with B; graph A’ comparable with B’). Thus, the obtained data suggest that the ATP/ADP ratio, per se, does not remarkably influence the studied reaction. However, the increase in total AXP concentration was found...
to inhibit the formation of valproyl-dephCoA. This effect was strengthened when phosphate was absent, but at [AXP] ≥ 5 mM, the obtained rate tended toward a similar residual value. In the absence of AXP, the rate of valproyl-CoA dephosphorylation was clearly decreased by the presence of 20 mM KPi in the medium, and in this condition ([AXP]/H11032 0 and [KPi]/H11005 20 mM, i.e., graphs A’ H11032 and B’ H11032), the increase of [AXP] only slightly affected those values.

**Metabolism of Valproyl-dephosphoCoA in RLM Homogenate.**

The possible backward reaction, using pure valproyl-dephCoA as starting material, was also investigated and the formation of valproyl-CoA was checked.

The decrease in valproyl-dephCoA (0.1 mM) was first studied in SEM buffer followed by HPLC analysis. The area of the valproyl-dephCoA peak remained practically unchanged, even after 2 h of incubation at 37°C. However, in the presence of a mitochondrial homogenate, a progressive disappearance of the substrate was observed with time (50% in 2 h), which was not affected by ATP (0–5 mM) (data not shown). The HPLC monitoring of these samples never revealed traces of valproyl-CoA. Whether this 50% decrease of valproyl-dephCoA (within 2 h) is due to a specific enzymatic reaction, to its hydrolysis, or to any other metabolic pathway (further β-oxidation) remains to be established.

**Discussion**

The present results provide evidence that the metabolism of valproic acid in intact RLM leads to the formation of a new metabolite identified as valproyl-dephosphoCoA. This evidence was built on 1) its formation from both [4,5-^3H_2]VPA and [2-^3H]VPA incubated with intact RLM (Fig. 2); 2) its dual detection in HPLC analysis as a labeled and UV-absorbing compound; and 3) its unequivocal identification by mass spectrometry (Fig. 3). The estimated intramitochondrial concentrations of valproyl-dephCoA (in respiratory states 3 and 4) are comparable to those of 3-oxovalproyl-CoA and, thus, 5-fold lower than the most abundant metabolite, valproyl-CoA (Silva et al., 2001).

The mechanism proposed for the formation of valproyl-dephCoA is a direct one-step conversion from valproyl-CoA by dephosphorylation (Fig. 1). This reaction may involve a phosphate release from ribose-3-phosphate in the adenosine moiety of the CoA group. The evidence in support of this hypothesis comes from the present results of studies with rat liver homogenate and valproyl-CoA. Our data suggest that this reaction can be catalyzed by a mitochondrial phosphatase, which is susceptible to inhibition or activation in different experimental conditions. The increased rate of valproyl-CoA dephosphorylation obtained in the absence of exogenous ATP can be a direct measure of the phosphatase activation state. Absence or lower concentrations of exogenous KPi as well as low values of the AXP pool also led to an enhanced dephosphorylation. These results suggest that any intramitochondrial depletion of these components (KPi, ATP, AXP) will increase the conversion of valproyl-CoA into valproyl-dephCoA. Figure 7 clearly shows that AMP and ADP are equally effective compared with ATP in preventing the dephosphorylation of valproyl-CoA. In fact, these effects are similar to that of KPi. Apparently, the need of mitochondria for phosphate is so significant that it will be stripped of any available substrate, being a CoA ester or a high energy phosphate such as ATP.
CoA has not been established yet. However, we consider it likely that such formation takes place, especially when changes in the AXP/P ratios occur. The biological significance of increased levels of dephospho-CoA esters has to be established. This may eventually lead to a better understanding of some of the unwanted effects of valproate, since mitochondria are a critical target for toxicity.

References


Address correspondence to: Dr. Margarida F. Silva, Centro de Patogênese Molecular - Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal. E-mail: mfsilva@tif.lisboa.pt.