

In vitro evidence of inhibition of mitochondrial protease processing by HIV-1 protease inhibitors in yeast: a possible contribution to lipodystrophy syndrome

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Received 29 March 2002; received in revised form 24 May 2002; accepted 24 May 2002

Abstract

Highly active antiretroviral therapy has been associated with the emergence of lipodystrophy syndromes that have clinical features commonly seen in patients with mitochondrial dysfunction. The effect of therapeutic protease inhibitors (PIs) on mitochondrial function is unknown. Mitochondrial matrix space proteins possess an amino-terminal leader peptide that is removed by the mitochondrial processing protease (MPP). Lack of cleavage could result in non- or dysfunctional mitochondrial proteins. The effects of different PIs on protease processing using pure MPP or yeast mitochondria, recognized models for mammalian counterparts, were examined in vitro. Multiple PIs were found to inhibit MPP, evidenced by accumulation of immature pALDH and decreased levels of processed ALDH. Both indinavir and amprenavir at 5.0 mg/ml resulted in significant inhibition of MPP. Although inhibition of MPP was also observed with ritonavir and saquinavir, the inhibition was difficult to quantify due to background inhibition of MPP by DMSO that was required to solubilize the drugs for the in vitro studies. Indinavir was also shown to inhibit MPP within yeast mitochondria. Lack of processing may impair mitochondrial function and contribute to the observed mitochondrial dysfunctions in patients receiving HAART and implicated in antiretroviral-associated lipodystrophy. © 2002 Elsevier Science B.V. and Mitochondria Research Society. All rights reserved.

Keywords: Lipodystrophy; Mitochondria; Inhibition; Protease

1. Introduction

Highly active antiretroviral therapy (HAART), nucleoside reverse transcriptase inhibitors (NRTIs) in combination with protease inhibitors (PIs), has emerged as the standard of care in the treatment of HIV disease (Fauci et al., 2002). Implementation of

HAART resulted in dramatic declines in HIV morbidity and mortality (Palella et al., 1998) and improvement in immunologic function, as a consequence of better virologic control compared to that achieved historically.

HAART has also been associated with the emergence of lipodystrophy syndromes characterized by peripheral fat wasting, central adiposity, hyperlipidemia, insulin resistance and diabetes mellitus (Carr et al., 1998a,b; Mauss, 2000). Epidemiological attempts

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to attribute these syndromes to any one specific drug or class of drugs have been unsuccessful. However, it is clear that their emergence as distinct clinical entities was subsequent to the widespread use of PIs in clinical practice, implying that the combination of drug classes is somehow responsible. The new complications of antiretroviral therapy comprise a constellation of symptoms implicating mitochondrial involvement. While the mitochondrial toxicity due to inhibition of mitochondrial DNA polymerase gamma by NRTIs is well described (Lewis and Dalakas, 1995), the effect of PIs as a class on mitochondrial function is unknown.

Nuclear genes encode the majority of the proteins found in mitochondria. Many of these mitochondrial proteins are synthesized as precursors with an N-terminal extension of amino acids, called a leader sequence. These precursors are imported into the matrix space via the TIM/TOM translocation complex (Neupert, 1997). The leader peptide is subsequently cleaved in the matrix, by the mitochondrial processing protease (MPP), producing the mature protein. The chelating agent EDTA and synthetic peptides corresponding to a leader sequence have been shown to inhibit MPP (Waltner and Weiner, 1995).

HIV-1 protease is an aspartyl (carboxyl) protease. MPP is defined as a metalloprotease due to the presence of a zinc ion in its active site region. The active site of MPP is located in a large central cavity, situated between the α and the β subunits, that is lined with hydrophilic amino acids, including glutamate and aspartate residues from each subunit (Ito, 1999; Taylor et al., 2001). We postulated that MPP might have a similar mechanism of catalysis as HIV-1 protease, and therefore be inhibited by HIV-1 PIs. In this report we investigated the inhibition of yeast MPP by PIs, currently licensed for clinical use, as measured by the ability of MPP to process rat liver aldehyde dehydrogenase precursor (pALDH) to mature ALDH (ALDH). A preliminary report of this work has been presented in poster form at the 2001 Mitochondria meeting in San Diego, CA, USA (Zullo et al., 2001).

2. Material and methods

2.1. Import of preproteins into isolated mitochondria

Radiolabeled preproteins were synthesized in the

presence of [35 S] methionine using the TNT Quick Coupled transcription and translation system (Promega). Preproteins were incubated with yeast mitochondria (Glick and Pon, 1995) for 30 min at 30°C in import buffer (0.6 M sorbitol, 50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2.0 mM KH_2PO_4 and 1.0 mg/ml Fatty-acid free BSA, pH 7.0). The final volume of the import mixture was 50 μ l. Import reactions were performed as described (Hammen et al., 1996; Heard and Weiner, 1998). Briefly, after incubation at 30°C, 3 μ l proteinase K (2 mg/ml) was added to digest the preproteins that were not imported. Proteinase K digestion was terminated by the addition of 3 μ l Phenylmethylsulfonyl fluoride (PMSF) (100 mM). Mitochondria were then isolated by centrifugation at 4°C. Sodium dodecyl sulfate (SDS) treatment buffer was added to dissolve the pelleted mitochondria which were then subjected to separation on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Quantification of import was performed using the band intensities from the SDS-polyacrylamide gels that were analyzed by a PhosphorImager storage technology (Molecular Dynamics) as done previously (Hammen et al., 1996). Indinavir was incubated with mitochondria for 15 min on ice before the TNT synthesized pALDH was added to study its ability to inhibit import and processing. The level of import was defined as the ratio of the total counts of the protease-protected bands divided by the initial counts provided in the assay. Each experiment was reproduced at least three times.

2.2. In vitro processing peptidase activity

Alpha and β subunits of MPP were cloned from yeast genomic DNA. Primers were designed based on published sequences of both subunits (Geli, 1993). cDNAs of both subunits were cloned to the PET-19B vector (Novagen) and transformed to *Escherichia coli* BL21 (DE3). Proteins were expressed in *E. coli* with an N-terminal His tag and the subunits were purified separately with a Ni-column (Qiagen). Mixing of both purified subunits gave the active hetero dimeric MPP. A standard MPP assay consisted of 1 μ l of reticulocyte lysate containing [35 S] methionine labeled pALDH, 2 μ l of the MPP (0.4–0.6 μ g), 10 mM Hepes–KOH, 1 mM

dithiothreitol, and 0.1 mM MnCl₂ (with a final volume of 20 μ l) and was incubated for 30 min at 27°C. The reactions were terminated by the addition of an equal volume of SDS treatment buffer. For the inhibition of processing, various amounts of different drugs dissolved in processing peptidase assay buffer (above) were allowed to interact with MPP for 15 min on ice and then it was added to the processing assay. Incubations with translated pALDH were performed for 30 min under standard assay conditions. Samples were subjected to 10% SDS-PAGE and autoradiography. Protein amounts were quantified using densitometry (Hammen et al., 1996). Indinavir was solubilized at a concentration of 8 mg/ml in water after prolonged vortexing. Amprenavir, ritonavir and saquinavir were initially solubilized in DMSO. In the assay buffer, amprenavir was present at a concentration of 1 or 5 mg/ml in 15% DMSO. Ritonavir was present in the assay buffer at concentration of 0.4 mg/ml in 20% DMSO. Saquinavir was present in the assay buffer at a concentration of 2 or 20 mg/ml in 20% DMSO.

3. Results

3.1. Inhibition of MPP

To determine if known HIV-1 PIs could affect the processing of mitochondrial precursor proteins, their ability to inhibit purified MPP was investigated. Recombinant expressed yeast MPP was used in the assay. Indinavir, amprenavir, ritonavir, and saquinavir were used as PIs. First, MPP was incubated for 15 min with the drugs and then pALDH, synthesized in TNT, was added to the reaction and was incubated further for 30 min at 27°C. The ratio of the mature form to the precursor remaining was taken as a measure of the drug's ability to inhibit the protease.

3.2. Indinavir as an inhibitor

Indinavir was found to inhibit processing in a concentration dependent manner. Approximately 0.5 mg/ml was found to inhibit processing by 30%, as shown in lane 6, Fig. 1. In the presence of 5 mg/ml, the highest dose tested, greater than 90% of processing was inhibited, as noted by the near absence of the

band corresponding to the size of mature ALDH (lane 8, Fig. 1).

3.3. Amprenavir as an inhibitor

Amprenavir also was capable of inhibiting MPP. The solubility of the drug was poor, so a mixed solvent containing 15% by volume of DMSO was needed. The presence of the organic solvent inhibited MPP as can be seen when comparing lanes 2 and 3 in Fig. 2. Under the conditions of the experiment, approximately 70% of pALDH was processed in buffer compared to 50% in the mixed solvent. Though 1 mg/ml of drug was found to inhibit the processing, more inhibition was found when 5 mg/ml was used.

3.4. Ritonavir and saquinavir as inhibitors

Ritonavir proved to be highly insoluble. It was necessary to use a 20% DMSO volume fraction to achieve limited solubility. Unfortunately, this concentration of solvent drastically inhibited the ability of MPP to function. The highest concentration of ritonavir that could effectively be maintained in solution during the experiment was 0.4 mg/ml. The presence of this comparatively low concentration of drug coupled with high background inhibition by DMSO, made it difficult to observe any significant inhibition as illustrated in Fig. 3. Similar to ritonavir, saquinavir

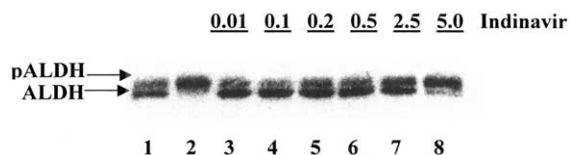


Fig. 1. Effect of indinavir on purified MPP. Inhibition of the processing enzyme (MPP) was measured in the presence and absence of indinavir. Mitochondrial processing peptidase was incubated with different concentrations of indinavir (0.01–5 mg/ml) on ice for 15 min. Then radiolabeled ALDH precursor (pALDH) was added to the assay buffer and incubation was carried out for an additional 30 min at 27°C. SDS-treatment buffer was added to terminate the reaction and samples were run on SDS-PAGE as described in Section 2. Lane 1 contains pALDH + MPP, lane 2 contains pALDH, lane 3 contains pALDH + MPP + indinavir 0.01 mg/ml, lane 4 contains pALDH + MPP + indinavir 0.1 mg/ml, lane 5 contains pALDH + MPP + indinavir 0.2 mg/ml, lane 6 contains pALDH + MPP + indinavir 0.5 mg/ml, lane 7 contains pALDH + MPP + indinavir 2.5 mg/ml, lane 8 contains pALDH + MPP + indinavir 5.0 mg/ml.

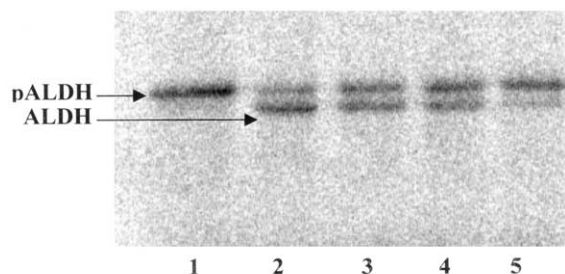


Fig. 2. Effect of amprenavir on purified MPP. Inhibition of the processing enzyme (MPP) was measured in the presence and absence of amprenavir. Inhibition of amprenavir on MPP was carried out as described in Fig. 1. The minor difference here was that the drug was solubilized in DMSO. Since MPP was partially inhibited by DMSO, a control reaction was performed in the presence of DMSO. Lane 1 contains pALDH, lane 2 contains pALDH + MPP, lane 3 contains pALDH + MPP (15% DMSO), lane 4 contains pALDH + MPP + amprenavir 1.0 mg/ml (15% DMSO), lane 5 contains pALDH + MPP + amprenavir 5.0 mg/ml (15% DMSO).

was equally insoluble and required 20% DMSO for limited solubility. Inhibition of MPP by saquinavir was observed at the highest concentration (20 mg/ml), but this proved to be only 10–12% over the high background inhibition by DMSO (Fig. 4). Due to the very low solubility of the ritonavir and saquinavir it is not possible to definitively state whether or not MPP inhibition occurred.

3.5. Inhibition in intact mitochondria

To ascertain whether the drug could be an inhibitor of MPP in mitochondria, a different assay was

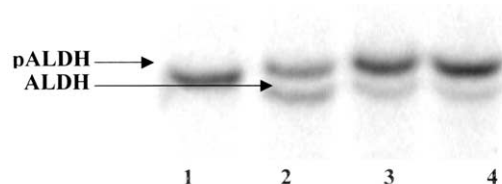


Fig. 3. Effect of ritonavir on purified MPP. Inhibition of the processing enzyme (MPP) was measured in the presence and absence of ritonavir. Inhibition of ritonavir on MPP was performed as described in Fig. 2. Ritonavir was solubilized in DMSO. Lane 1 contains pALDH, lane 2 contains pALDH + MPP, lane 3 contains pALDH + MPP (20% DMSO), lane 4 contains pALDH + MPP + ritonavir 0.4 mg/ml (20% DMSO).

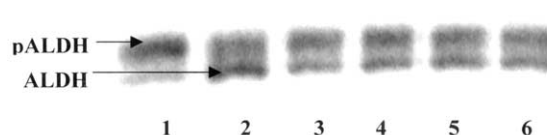


Fig. 4. Effect of saquinavir on purified MPP. Inhibition of the processing enzyme (MPP) was measured in the presence and absence of saquinavir. Inhibition of saquinavir on MPP was performed as described in Fig. 2. Saquinavir was solubilized in DMSO. Lane 1 contains pALDH, lane 2 contains pALDH + MPP, lane 3 contains pALDH + MPP + saquinavir 20 mg/ml (20% DMSO), lane 4 contains pALDH + MPP (20% DMSO), lane 5 contains pALDH + MPP + saquinavir 2.0 mg/ml (20% DMSO). Lane 6 contains pALDH + MPP (20% DMSO).

employed. Mitochondrial import assay was performed in the presence of indinavir. Mitochondria were incubated for 15 min with indinavir from 0 to 5 mg/ml concentrations prior to import reaction. TNT synthesized pALDH was then added to the drug treated mitochondria and the import reaction was carried out for 30 min at 30°C. The conversion of pALDH to mature ALDH is a measure of import and processing. As proteinase K was used to destroy unimported precursor protein, the presence of pALDH shows that it was imported but not processed in the presence of drug. It can be seen in Fig. 5 that 0.1 mg/ml indinavir has no effect on MPP processing (lane 3, Fig. 5). However, 0.5–2.5 mg/ml of drug inhibited the processing (lanes 4–6, Fig. 5). These concentrations of drug, though inhibiting the processing, did not inhibit the

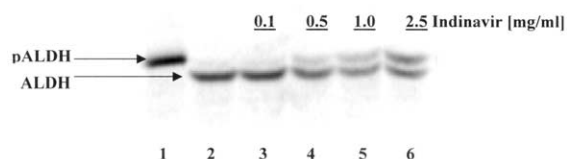


Fig. 5. Effect of indinavir on import and processing of pALDH in yeast mitochondria. To study the ability of the drug to inhibit MPP when it is in the matrix space an import assay was performed in the presence and absence of Indinavir. Mitochondria were incubated with different concentration of indinavir in import assay buffer for 15 min. Translated pALDH was then added to the mitochondria and import reaction was performed at 30°C for 30 min. Details of the import reaction were described in Section 2. Lane 1 contains pALDH, lane 2 contains pALDH + mitochondria, lane 3 contains pALDH + mitochondria + indinavir 0.1 mg/ml, lane 4 contains pALDH + mitochondria + indinavir 0.5 mg/ml, lane 5 contains pALDH + mitochondria + indinavir 1.0 mg/ml, lane 6 contains pALDH + mitochondria + indinavir 2.5 mg/ml.

mitochondrial import. However, when we used the drug at a concentration of 5 mg/ml, it inhibited the mitochondrial import (data not shown), so at that concentration of drug we could not compare the *in vitro* and *in vivo* effect on MPP.

4. Discussion

The pathogenesis of antiretroviral-associated lipodystrophy syndromes is unclear. Indirect data have led some authors to propose that mitochondrial dysfunction could play a role (Brinkman et al., 1999), while others suggest PIs might affect proteins involved in the regulation of lipid metabolism (Carr et al., 1998a,b). Since lipodystrophy did not emerge as a distinct clinical entity until after the introduction of PIs as part of combination therapy for HIV infection, it was initially presumed to be specifically due to HIV-1 PIs. However, lipodystrophy has been documented in patients who have never received PIs and many patients receiving PIs never develop lipodystrophy.

While the role of PIs in combination with NRTIs in the development of lipodystrophy are unclear, emerging evidence suggests that both classes of drugs are involved in the development of lipodystrophy and have direct effects on adipocytes. As a consequence, the interactions between these two drug classes may be the most powerful determinant of fat wasting and development of the lipodystrophy syndrome (Nolan et al., 2001). NRTIs have been documented to independently contribute to fat wasting (Mallal et al., 2000). This NRTI-induced predisposition to slowly progressive fat loss is markedly accelerated when PIs and NRTIs are combined. In addition, genetic and structural defects of mitochondria in adipose tissue have been documented in patients receiving NRTIs exclusively (Walker et al., 2002). In this study, mean mtDNA content was 39% lower in patients with lipodystrophy compared to those without lipodystrophy. While no point mutations or deletions in mtDNA were documented, adipocytes displayed ultrastructural abnormalities suggestive of mtDNA cytopathies. In studies of preadipocyte cell lines, both PIs and to a lesser extent NRTIs were documented to interfere with adipocyte differentiation (Roche et al., 2002). More importantly, the effects produced by combinations of the two classes of drugs were different from those elicited

by individual drugs separately, underscoring the reciprocal nature of drug interactions that may influence the development of lipodystrophy and validating our rationale for conducting these investigations.

Studies continue to document the central role of mitochondria as a marker of nucleoside toxicity and their involvement in antiretroviral-related lipodystrophy. Alterations in both mtDNA levels and mitochondrial function have been found. MtDNA levels are significantly reduced in patients with symptomatic NRTI-related hyperlactatemia (Cote et al., 2002). In addition to reductions in mtDNA content, abnormalities of mitochondrial structure and respiratory chain dysfunction have been described in patients with lipodystrophy (Zaera et al., 2001; Shikuma et al., 2001). In animal models, the NRTI stavudine has been demonstrated to have differential effects on both mtDNA depletion and fatty acid oxidation in lean and obese mice (Gaou et al., 2001).

We have demonstrated that PIs are capable of inhibiting recombinant yeast MPP and impairing the cleavage of mitochondrial leader sequences, resulting in unprocessed, mitochondrial preproteins in the mitochondrial matrix. Our system is an accepted model for mammalian MPP function (Hammen et al., 1996). It was found that both indinavir and amprenavir clearly inhibit processing catalyzed by purified MPP and the data suggest that saquinavir is also capable of inhibition. The effects of ritonavir and saquinavir on MPP could not be adequately assessed due to intractable solubility issues under the experimental conditions employed. Additionally, indinavir also proved to be an inhibitor of the MPP enzyme in intact yeast mitochondria, providing further support for a plausible effect of PIs on mitochondrial function *in vivo*. We did not determine if the drugs used to inhibit HIV-1 protease would inhibit mitochondrial function such as respiration or metabolism, although preliminary experiments in yeast culture by our collaborators suggest that mitochondrial function is compromised (Von Borstel et al., in preparation). Respiratory chain dysfunction associated with multiple mitochondrial DNA deletions has been reported in at least one patient with HAART-related lipodystrophy (Miro et al., 2000). Biochemical analyses of mitochondrial respiratory function revealed decreased complex III and IV activities in both skeletal muscle and lymphocytes but was not analyzed in adipocytes.

Multiple deletions of mitochondrial DNA (mtDNA) were found in skeletal muscles and adipocytes, but not in lymphocytes. Another recent study documented structural muscle abnormalities, mitochondrial respiratory chain dysfunction or mtDNA deletions in all seven patients examined with HIV lipodystrophy, further suggesting that mitochondrial dysfunction plays a role in the development of antiretroviral therapy-related lipodystrophy (Zaera et al., 2001).

There is no published information as to what degree of MPP inhibition would correlate with diminished function or mitochondrial damage and depletion. However, it is known that deletion of MPP from yeast prevents the cell from growing aerobically (Adamec et al., 1999). It has also been shown that some mitochondrial enzymes are active when in the precursor form, including ALDH (Jeng and Weiner, 1991). Some mitochondrial enzymes are not fully active if they are not processed (Geli, 1993). However, it was recently found that the non-processed pALDH was rapidly degraded in mitochondria (Yang and Weiner, submitted). If PIs are inhibiting mitochondrial precursor processing in patients *in vivo*, then the subsequent mitochondrial impairment that might be anticipated is consistent with the clinical manifestations documented among affected patients.

The MPP is a metallo-protease and the only known inhibitors are metal chelators or peptides corresponding to leader peptides (Luciano and Geli, 1996). It is striking to note that both MPP and HIV-1 protease utilize the carboxylic amino acid side chains as the catalytic active site. The MPP active site utilizes a glutamic acid residue in the catalytic removal of leader peptide (Braun and Schmitz, 1995), while the HIV-1 protease utilizes aspartic acid residues in the catalyzed scission of the viral protein. In our studies, significant inhibition of MPP was only seen at supra-physiologic concentrations of drug: 2.5 and 5.0 mg/ml, 1.0 and 5.0 mg/ml and 20 mg/ml for indinavir, amprenavir, and saquinavir respectively. It can be argued that because such high PI concentrations were necessary to inhibit MPP that these findings may be of limited physiologic importance. Although the concentrations of drug needed to induce inhibition were high, chronic administration of these drugs might result in their accumulation over time within the mitochondrial matrix, thus facilitating inhibition. Other drugs have been documented to concentrate in

mitochondria and inhibit oxidation and respiratory function (Berson et al., 2001). Additionally, drugs utilized in these experiments were returned, prescribed clinical lots and thus carrier substances were present as well as the actual drugs. Further work should use pure, pharmaceutical grade PIs.

Most PIs are metabolized by the cytochrome P450 system in the liver, while the constellation of clinical symptoms associated with lipodystrophy is generally manifest in other tissues. It is cogent to note that PIs are highly hydrophobic, and concentration in lipidaceous tissues might be expected. It is unknown whether sequestration of PIs by mitochondria occurs, but our results strongly suggest that this should be explored in future investigations. High mitochondrial concentrations of PIs might be attained *in vivo* if preferential sequestration of PIs in the mitochondria occurred as a consequence of prolonged, sustained exposure to PIs in patients receiving HAART. If so, it is feasible that the effective concentration of PIs in the highly proteinaceous mitochondrial matrix could approach the concentrations that inhibit MPP described here. The possibility of PI sequestration in mitochondria is also consistent with recent evidence documenting an increased risk for lipodystrophy associated with the duration of exposure to antiretroviral therapy (Martinez et al., 2001). As noted previously, there is now direct evidence of decreased mtDNA in subcutaneous adipose tissue of HIV-infected patients with peripheral fat wasting lipodystrophy (Shikuma et al., 2001). While the authors of this study were focused on investigating the effects of NRTI-containing HAART regimens on mtDNA content in fat tissue, their results do not preclude a possible contributory effect of PIs in diminishing mtDNA as a consequence of MPP inhibition demonstrated here.

Mitochondria in patients receiving HAART are likely to be compromised, given both the known mitochondrial toxicity of NRTIs and the stress of HIV infection. Additional prospective data support a contributory role for NRTIs in the development of antiretroviral therapy-associated lipodystrophy (van der Valk et al., 2001). Hence, underlying mitochondrial impairment due to NRTIs might predispose to further dysfunction associated with lack of mature mitochondrial proteins as a consequence of PI inhibition of MPP. Our results provide a potentially unifying theme in the multifactoral pathophysiology of

lipodystrophy, given the implicated involvement of mitochondrial dysfunction, NRTIs, PIs, and the duration of antiretroviral exposure. Finally, the clinical relevance of this mechanism as a potential contributory factor in the development of lipodystrophy is underscored by the epidemiologic evidence documenting the emergence of lipodystrophy syndromes following the widespread implementation of PI-containing HAART regimens as the standard of HIV care.

Future investigations should examine mitochondrial matrix proteins from biopsied adipose tissue in lipodystrophy patients receiving PI-containing HAART regimens. We hypothesize, based on our current *in vitro* and *in organello* results that *in vivo* evidence of inhibition of MPP will be found. Additional studies should be conducted concurrently to determine if there is evidence of PI accumulation or sequestration by mitochondria and correlated with levels of mitochondrial respiratory chain function. Recently, reported techniques describing direct analysis of mitochondrial toxicity of antiretroviral drugs (Foli et al., 2001) should be utilized to examine PIs. We also suggest that PIs in development should be screened for inhibition of MPP in addition to developing new HIV-1 PIs that do not affect MPP.

5. Disclaimer

The identification of any commercial product or trade name does not imply endorsement or recommendation by either the National Institutes of Health or the National Institute of Standards and Technology.

Acknowledgements

This work was supported in part by grants from the Institute on Alcohol and Alcoholism (AA10795) and General Medicine (GM53269) of the National Institutes of Health. This is journal paper number 16673 from the Purdue University Agriculture Experiment Station.

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