Palm Fruit Juice Mitigates AZT Mitochondrial Genotoxicity and Dose-Dependent Cytotoxicity

Osborne AE1, Sanchez JA2, Solomon M2, Stopa A1, Wangh LJ1, Sambanthamurthi R2 and Hayes KC1

1Department of Biology, Brandeis University, Waltham, MA, 02454, USA
2Malaysian Palm Oil Board, Kajang, Selangor, Malaysia

Corresponding author: Sanchez JA, Department of Biology, Brandeis University, Waltham, MA, 02454, USA, Tel: 1-781-736-3111; Fax: 1-781-736-3107; E-mail: sanchez@brandeis.edu

Received date: September 18, 2014; Accepted date: December 16, 2014; Published date: December 20, 2014

Abstract

Chronic use of 3′-Azido-3′-deoxythymidine (AZT) to treat HIV/AIDS causes mitochondrial dysfunction and the accumulation of mitochondrial mutations. These toxicities have been attributed to increased oxidative damage, among other mechanisms. Palm fruit juice (PFJ), also known as oil palm phenolics (OPP), is a water soluble by-product of oil extraction from the fruit of the oil palm (Elaeis guineensis) that is rich in antioxidants and other phytochemicals. The capacity of PFJ to mitigate AZT mitochondrial genotoxicity (mutagenesis) as well as dose-dependent cytotoxicity was measured in cultured HepG2 cells. In the presence of PFJ, AZT-induced mutations were 35% the number of mutations observed in samples treated with AZT alone in the three regions of the mitochondrial genome examined (HV2, CO2, and ND1). Co-treatment with PFJ increased cell survival in the presence of increasing doses of AZT by up to 350%. These effects were not due to degradation or inactivation of AZT by PFJ. The discovery of the mitigating effects of PFJ provides a potential means of ameliorating AZT-induced mutations and possibly other long-term negative side effects of long-term AZT use.

Keywords: Mitochondria; LATE-PCR; Light-on/Lights-off probes; AZT toxicity; Palm fruit juice; Oil palm phenolics; Mutational load

Introduction

The nucleotide reverse transcriptase inhibitor (NRTI) 3′-Azido-3′-deoxythymidine (AZT, zidovudine) is widely used in the developing world to treat HIV/AIDS. AZT treatment, however, results in toxic side effects, including skeletal and cardiac myopathies, hyperlactatemia, and peripheral neuropathy [1-4]. Chronic AZT therapy is also associated with an increased incidence of mitochondria-related diseases such as type 2 diabetes and neurological disorders [5-7]. These side effects are secondary to the anti-retroviral activity of the drug and are consistent with AZT damage leading to mitochondrial dysfunction [8-10]. Because of its toxicity, AZT has been replaced by other less harmful NRTIs in most developed countries. Nonetheless, AZT remains an integral component of highly active anti-retroviral therapy (HAART) in the rest of the world. Mitochondrial damage and dysfunction resulting from AZT treatment remain of great concern in the treatment of HIV/AIDS.

The exact mechanism of AZT toxicity is not known. AZT toxicity could be due to mitochondrial dysfunction resulting from increased oxidative stress and/or mitochondria DNA mutations. Many clinical and experimental studies have shown mitochondrial dysfunction and increased oxidative stress following AZT treatment both in vitro and in vivo [11-13]. Consistent with these observations, AZT toxicity in cell culture, mice, and humans has been mitigated by co-treatment with individual antioxidants (vitamin C, vitamin E, resveratrol, etc.) [14-17]. There is now direct evidence that AZT causes mitochondrial DNA mutations [18-20].

This report describes the use of an antioxidant, palm fruit juice (PFJ), to mitigate AZT-induced mitochondria mutations and dose-dependent cytotoxicity in HepG2 cells, the experimental system in which mitochondrial mutagenesis by AZT was recently reported [18]. Palm fruit juice is a water soluble by-product of oil extraction from the fruit of the oil palm (Elaeis guineensis) that is rich in antioxidant phenolics [20,21]. Palm fruit juice has high scavenging activity against hydrogen peroxide, the main contributor to reactive oxygen species (ROS) produced in mitochondria [20,22,23]. Palm fruit juice also exhibits various protective biological effects, including anti-atherogenic, anti-inflammatory, anti-diabetic, and anti-neoplastic properties [24-31]. For example, dietary supplementation with PFJ deters the progression of type 2 diabetes, a disease associated with both mitochondrial dysfunction and chronic AZT treatment [29,31]. The results of the present study reveal that PFJ mitigated both AZT-mutagenesis and dose-dependent cytotoxicity.

Materials and Methods

Detection of Mutations after AZT and PFJ Treatment for 30 Days - Culture Conditions and DNA Preparation

Cell culture conditions and preparation of the mitochondrial DNA were the same as previously reported [18] except that HepG2 cells were also cultured in the presence or absence of 25 μg gallic acid equivalents (GAE)/ml PFJ (a gift from the Malaysian Palm Oil Board). Twenty-five micrograms GAE per millilitre PFJ was the highest concentration that permits normal cell growth in conventional Eagle’s Minimum Essential Medium (EMEM, ATCC Manassas, VA) used in the original experiments where AZT was shown to be mutagenic to mitochondria [18]. Buffered culture medium employed in the cytotoxicity study (see Supplemental Data) was not used for these
experiments to preserve the original culture conditions used to identify AZT-induced mutations [18]. The thirty-day incubation time for the genotoxicity study described here and in the original study [18] was based on reports that mitochondrial dysfunction in HepG2 cells caused by the physiologically relevant concentrations of AZT used in our study (7 uM) requires long-term exposure (4 weeks) to develop [11].

Mitochondrial DNA was prepared as previously reported [32]. Briefly, one microliter of cultured cells (roughly 1000 cells) was lysed in 14 µl of Quantilyse [33]. Samples were stored at -20°C.

Cytotoxicity Tests

HepG2 cultures were incubated with increasing concentrations of AZT (0 µM, 10 µM, 30 µM, 50 µM, 70 µM, 100 µM) in the presence or absence of 75 µg GAE/ml PFJ in buffered media (see Supplemental Information). Seventy-five micrograms GAE per millilitre PFJ was used for these studies because it was the highest concentration of PFJ that did not arrest the growth of HepG2 cells after 25 day culture under buffered culture conditions (Supplemental Figure 1). Growth arrest observed at concentrations above 75 µg GAE/ml PFJ is characteristic of the known cytostatic activity of PFJ against neoplastic cells such as HepG2 cells [34].

LATE-PCR Lights-On/Lights-Off Probe Assay for Mutation Detection

Reagents, primers, probes, and reaction conditions

Lyzed DNA samples were first diluted to the digital or near-digital level (1-5 copies) as described in Osborne et al. [32]. To avoid contamination, all PCR preparation was conducted in an amplicon-free hood in a clean room, all targets were kept separate from PCR reagents at all times, and PCR machines were kept in a separate room from the laboratory space as far away from the clean room as possible. PCR amplicons and products were never handled in the same laboratory space.

Primers, probes and amplification conditions used for the triplex LATE-PCR assay for mutation analysis of the mitochondrial hyper variable 2 (HV2) region of the D-Loop, the cytochrome c oxidase subunit 2 (CO2) gene, the NADH dehydrogenase, subunit 1 (ND1) gene were as previously described [18]. A single master mix was used for all experiments to ensure the reproducibility of the fluorescent signatures.

Mutation analysis

Mutations were scored as described by Osborne et al. Briefly, sets of Lights-On/Lights-Off probes spanning the length of each amplicon were used to generate fluorescent signatures characteristic of each amplicon sequence. Changes in a fluorescent signature, i.e., shifts to lower or higher temperature or appearance or disappearance of peaks and valleys, relative to a reference signature were indicative of mutations. To obtain reference signatures for normal sequences, bulk reactions containing over 1000-copies of mtDNA each were amplified and the resulting fluorescent signatures were averaged together (for a more detailed discussion of fluorescent signature analysis see Osborne et al.). Shifted signatures that had more than two temperature points outside the confidence intervals of the reference signature were considered a true shift due to mutation rather than noise. These objective criteria for scoring mutations eliminated subjective bias during mutation assessment [18].

Measurement of mtDNA copy number

Measurement of mtDNA copy number was carried out as previously reported in Osborne et al. [18].

Statistical analysis

Statistical analysis of the cytotoxicity and genotoxicity of the data was carried out using Pearson’s chi-square test. The Pearson’s chi-square test establishes whether there is a significant difference between the expected and observed frequencies in one or more of the treatment categories. The test was performed using 95% significance (p-value of less 0.05).

Results

Palm fruit juice mitigated AZT-induced mtDNA mutations

In order to investigate whether increased oxidative stress caused by AZT leads to mtDNA mutations, AZT-induced mutations were measured in HepG2 cells treated for 30 days with 7 µM AZT in the presence and absence of 25 µg GAE/ml PFJ. Seven micromolar AZT is the peak plasma concentration in AZT-treated individuals previously found to induce mutations in HepG2 cells [18]. Three regions from the mitochondrial genome, HV2, CO2, and ND1, were examined for

Figure 1: Palm Fruit Juice ameliorated AZT-induced mtDNA mutations. Thirty days of AZT treatment (7 µM) also resulted in an increase in mutations in the mitochondrial genome, while co-treatment with 25 µg GAE/ml PFJ reduced the number of mutations. Treatment with PFJ alone did not cause an increase in mutations. White=Non-treated control, Black=AZT treated, Grey=PFJ treated, and Striped=AZT and PFJ treatment. *represents those samples that had a p-value of 0.05 or less.

Cells were grown in 6-well plates at 37°C and 5% CO₂ in EMEM supplemented with 25 mM HEPES, 10% fetal bovine serum (BioWest), 50 units/mL Penicillin G, 50 units/mL Streptomycin, and 0.25 µg/mL Amphotericin B (HyClone Antibiotic/Antimycotic Solution 100X). Media and additives were replenished every other day. Palm fruit juice was sterilized through a 0.2 µm filter before addition to the media. Cell cultures were initially seeded with 1x10⁵ cells/ml. Every 3-4 days cells were trypsinized and stained for membrane integrity using Trypan Blue (Sigma Aldrich, St. Louis, MO). The cells were then counted to measure cell growth and re-plated in fresh media at 1x10⁵ cells/ml. Experiments were repeated three times.
mutations using a multiplex LATE-PCR Lights-On/Lights-Off assay at the digital or near digital levels [18]. A total of 352 wells with 1-5 genomes of mtDNA (an estimated 1,056 amplicons total) were evaluated.

AZT treatment alone caused up to a 9-fold increase in the number of random mutations in all three target genes compared to non-treated samples (p-values of 8×10⁻⁶, 0.0003, and 0.0008 for HV2, CO2, and ND1 respectively; Figure 1). Palm fruit juice treatment alone was not mutagenic (Figure 1). Co-treatment with 7 μM AZT and 25 μg GAE/mL PFJ resulted in 35% the number of random mutations caused by AZT alone (p-values of 0.005, 0.008, and 0.007 for HV2, CO2, and ND1 respectively; Figure 1). Measurements of the ratio of nuclear to mitochondrial genomes in PFJ and AZT co-treated samples showed that PFJ did not reduce the number of mitochondrial mutations by simply increasing the number of mitochondrial genomes (data not shown). These results demonstrate that PFJ ameliorated the mutagenic activity of AZT.

Palm Fruit Juice mitigated dose-dependent AZT cytotoxicity in HepG2 cells

To investigate whether PFJ also mitigates AZT dose-dependent cytotoxicity, HepG2 cultures were incubated with various concentrations of AZT (0 μM, 10 μM, 30 μM, 50 μM, 70 μM, 100 μM) in the presence or absence of 75 μg GAE/mL of PFJ. Increasing concentrations of AZT from 0 μM to 100 μM resulted in a progressive increase in cell death after 6 days in culture (Figure 2). Treatment with 100 μM AZT declined cell viability by 90%. Addition of 75 μg GAE/mL PFJ resulted in increased protection against AZT-induced cell death from a minimal 12% improvement at 30 μM AZT to about 350% improvement at 100 μM AZT (p-values of 0.04 and 0.01) (Figure 2). These results demonstrate that inclusion of PFJ in the culture medium mitigated AZT’s dose-dependent cytotoxicity.

Palm Fruit Juice did not degrade AZT

A possible explanation of the protective effects of PFJ on mutagenesis and cytotoxicity is that PFJ simply degraded AZT. This was not the case because day ten cultures showed that AZT modulated the cytostatic activity of PFJ. In the absence of AZT 75 μg GAE/mL PFJ did not arrest HepG2 cell growth after 25 days in culture (Supplemental Figure 1). In contrast, co-treatment with AZT and PFJ arrested cell growth after ten days in culture, although cells remained viable (Table 1). These results showed that PFJ did not inactivate AZT and that the protective effect of PFJ were due to the activity of PFJ within HepG2 cells.

Table 1: High concentrations of AZT enhance the cytostatic effect of 75 μg GAE/ml PFJ.

Day 6 culture cells were trypsinized and plated in fresh culture media with various concentrations of AZT in the absence or presence of 75 μg GAE/ml PFJ at an initial concentration of 1×10⁵ cells/ml. At day 10, cells were trypsinized again, stained with Trypan Blue, and counted four times per culture to measure the total concentration of cells. The results show that the same concentration of cells plated on Day 6 was recovered on Day 10 for 70 μM AZT and 100μM AZT co-treated with 75 μg GAE/ml PFJ and that cells remained viable. This phenomenon is characteristic of the cytostatic effect of 75 μg GAE/ml PFJ on HepG2 cells observed after Day 20 culture in the absence of AZT (see Supplementary Figure 1) but occurred 15 days earlier. These data indicate that high concentrations of AZT enhanced the cytostatic effect of 75 μg GAE/ml PFJ and that growth-arrested cells in the presence of PFJ continued to be protected from AZT cytotoxicity. In the presence of lower concentrations of AZT (30 μM and 50 μM), 75 μg GAE/ml PFJ exhibited slower cell growth suggestive of a partial PFJ cytostatic effect. *=cells displayed an aberrant morphology (Figure 1).

Discussion

The results presented here demonstrate that addition of PFJ mitigated both AZT-induced mitochondrial mutagenesis and dose-dependent cytotoxicity. Since AZT toxicity is related to mitochondrial dysfunction [8-10], the discovery that PFJ mitigated the genotoxic and cytotoxic effects of AZT suggests that PFJ exerts a protective effect on mitochondria. These studies expand the range of protective effects associated with PFJ [24-30].

Protective effects of PFJ against AZT mutagenesis

AZT treatment increases oxidative stress in cells and causes mutagenesis and dose-dependent cytotoxicity, but the biochemical basis for these phenomena remains unclear. Since PFJ is rich in
antioxidants and many antioxidants are known to modulate Reactive Oxygen Species (ROS) [23] it is logical to postulate that PFJ suppresses mutations caused by ROS. However, PFJ only reduced the number of AZT-induced mutations by 65%, suggesting that only a fraction of the mutations arise because of an increase in ROS and that the AZT also acts by ROS independent mechanisms. In fact, two hypotheses have been proposed to explain AZT-induced mutagenesis. Mutations might arise from damage of mitochondrial DNA by ROS generated by AZT [35-38]. Alternatively, mutations may be due either to deleterious interaction between AZT and the mitochondrial DNA polymerase or to changes in nucleotide pools, either of which could alter the fidelity of mtDNA replication [39,40]. It is also possible that the highest permissible concentration of PFJ in non-buffered media has a limited antagonistic capacity.

Regardless of which mechanism is operative, AZT-induced DNA damage likely results in damage to mitochondrial proteins and this, in turn, probably further increases ROS production and accelerates the processes of mitochondrial DNA damage and dysfunction. Thus, independently of mechanism, the functional benefit of PFJ can be viewed as delaying the accumulation of mitochondrial DNA damage and the rate at which mitochondrial dysfunction is manifest.

**Protective effects of PFJ against AZT cytotoxicity**

The protective effects of antioxidants such as PFJ against AZT cytotoxicity has not been widely studied [15,41]. Palm fruit juice may attenuate AZT cytotoxicity by countering increased oxidative stress. Given the complex composition of PFJ [42,43] a simple alternative hypothesis would be that some component(s) of PFJ might inactivate AZT in the culture media. The fact that AZT enhanced the cytostatic activity of PFJ argues against PFJ degrading AZT. Independent studies also show that AZT remains active in the presence of PFJ [23]. The cytostatic activity of PFJ appears to arise from changes in gene expression that lead to G1/S phase arrest [44]. Future studies will address how AZT interacts with cells to enhance the cytostatic effects of PFJ and how cell cycle arrest prevents AZT-induced cell death.

**Significance**

In contrast to other antioxidants used to mitigate AZT toxicity (such as vitamin E and resveratrol [14,17]) which are defined chemical species, PFJ is a complex mixture of chemicals. The experimental system described here could be used to identify the component(s) of PFJ responsible for the range of protective effects observed. The biomarkers used to evaluate the protective effects of PFJ (cytotoxicity and mitochondrial genotoxicity) could be used to assess other drugs and naturally derived phenolics for their effects on mitochondria.

The earlier findings that AZT treatment causes mutagenesis of mtDNA suggests that patients chronically treated with this drug as part of HAART might be experiencing continuous damage to their mitochondrial genomes [18,20,45]. Accumulation of mtDNA damage over time may explain the increased incidence of mitochondrial-related diseases observed in these patients. The discovery that PFJ mitigates AZT toxicity suggests a possible approach to ameliorate or significantly delay the toxic side effects of AZT affecting millions of people.

**Acknowledgements**

This work was funded in part by the Malaysian Palm Oil Board to K.C.H and L.J.W. The Malaysian Palm Oil Board had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

**References**

neurochemical and behavioral effects in a mouse model: protection by L-
Increases mtDNA Mutations in HepG2 and CCR5-1112Sk Cells. J AIDS
Accumulation of Mitochondrial DNA Mutations in Human Immunodeficiency Virus–Infected Patients Treated with Nucleoside-
Analogue Reverse-Transcriptase Inhibitors. Am J Hum Genet 72:
549-560.
al. (2011) Mitochondrial aging is accelerated by anti-retroviral therapy
during the clonal expansion of mtDNA mutations. Nat Genet 43:
806-810.
Diabetes and mitochondrial dysfunction in Alzheimer’s disease. J
(2014) Use of Palm Fruit Juice Shows that Oxidative Damage is not a
Major Contributor to AZT-Induced Mitochondrial Mutations.
isolated from oil palm black liquor waste. Environ Toxicol Pharmacol
36: 135-141.
Oil palm phenolics confer neuroprotective effects involving cognitive and
Oil palm phenolics attenuate changes caused by an atherogenic diet in mice.
TG, et al. (2011) Oil palm vegetation liquor: a new source of phenolic
Differential transcriptomic profiles effected by oil palm phenolics
indicate novel health outcomes. BMC Genomics 12: 432.
al. (2011) Positive outcomes of oil palm phenolics on degenerative
development of Type 2 diabetes in the Nile rat 24: 1945-1952.
diabetic effects of palm fruit juice in the Nile rat (Arvicanthis niloticus).
Journal of Nutritional Science 3.