Journal of
AIDS & Clinical Research
ISSN: 2155-6113

Osborne et al., J AIDS Clin Res 2015, 6:4
DOI: 10.4172/2155-6113.1000441

Research Article Open Access

Oxidative Damage is not a Major Contributor to AZT-Induced Mitochondrial Mutations

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Abstract

Addition of clinically-relevant levels of 3′-Azido-3′-deoxythymidine (AZT) to cultured HepG2 cells increases the number of reactive radical species (reactive oxygen and nitrogen species [ROS and RNS]) as well as random mutations in mitochondrial DNA (mtDNA). Co-treatment of AZT-exposed cells with palm fruit juice (PFJ) mitigates AZT mutagenesis. These findings suggest that AZT-dependent mtDNA damage resulted from increased reactive species and that PFJ, a known anti-oxidant, mitigated such damage by decreasing the levels of these species. The present report tests the predictions that (1) PFJ mitigates AZT mutagenesis by reducing the burden of AZT-induced reactive species, and (2) AZT-induced mutations in mtDNA should predominantly consist of G → T and C → A substitutions characteristic of DNA oxidative damage. Levels of reactive species and mitochondrial mutagenesis were measured in HepG2 cells exposed to AZT in the presence or absence of PFJ. Controls experiments showed that PFJ in HepG2 cells exhibited strong scavenging activity against hydrogen peroxide-induced ROS, the main reactive species generated by dysfunctional mitochondria. Despite this strong antioxidant activity, PFJ did not decrease AZT-induced reactive species at a concentration that mitigated mtDNA mutations. Consistent with this observation, the spectrum of AZT-induced mutations did not fit the spectrum expected from direct mtDNA oxidative damage. Instead, the spectrum obtained was consistent with the majority of mutations (80%) arising from mitochondrial DNA polymerase errors induced by AZT. These observations suggest that oxidative damage was not the major contributor to AZT-induced mutations.

Keywords: Mitochondria; AZT toxicity; Palm fruit juice; Oil palm phenolics; Antioxidant; Mitochondrial DNA mutations

Introduction

The nucleotide reverse transcriptase inhibitor 3′-Azido-3′-deoxythymidine (AZT, zidovudine) is a key drug used to treat HIV/AIDS in many countries of the developing world. AZT treatment, however, causes both short and long term toxic side effects (skeletal and cardiac myopathies, hyperlactatemia, peripheral neuropathy, increased incidence of diabetes and neurological disorders). These pathologies are consistent with AZT treatment leading to mitochondrial dysfunction and increased oxidative stress [1-3]. AZT treatment also results in the accumulation of random mutations in mitochondrial DNA (mtDNA) [4]. Mitochondrial dysfunction due to these mutations may further increase oxidative damage, initiating a feedback loop of more mutations and further oxidative damage leading to disease.

AZT may cause mitochondrial mutations and dysfunction by increasing the levels of reactive species (reactive oxygen and nitrogen species [ROS and RNS]) within mitochondria [5-9]. These reactive species oxidize DNA leading to the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), 8-nitro-7,8-dihydro-2'-deoxyguanosine (8-nitro-dG), and other less frequent oxidation products. Mis-pairing of 8-oxo-dG and 8-nitro-dG results in G → T and C → A nucleotide substitutions characteristic of oxidative damage [10-14].

The work presented here tested the hypothesis that oxidative damage triggered by AZT may be a primary cause of AZT-induced mutations in mtDNA. This hypothesis is supported by our observation that addition of palm fruit juice (PFJ) to AZT-treated cultures reduced the number of drug-induced mtDNA mutations. 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 and other phytochemicals [15]. In particular, PFJ exhibits a high scavenging activity for hydrogen peroxide, the main reactive oxygen species produced in excess by defective mitochondria [16]. Similarly, individual antioxidants such as resveratrol, vitamin C, and vitamin E have been shown to mitigate mitochondrial dysfunction due to AZT-induced oxidative stress *in vitro* and *in vivo,* although those studies did not measure mtDNA mutations associated with oxidative stress [9,17].

The experiments described here were designed to test two specific hypotheses. First, PFJ mitigation of AZT-induced mutations should correlate with reduced oxidative stress. Second, AZT-generated mutations should include excess G → T and C → A substitutions characteristic of oxidative damage. Despite demonstrating the strong antioxidant activity of PJF against hydrogen peroxide-induced ROS, neither of the above predictions proved true. Thus, the results question whether oxidative stress is the main driver of AZT-induced mutations.

Materials and Methods

Measurement of reactive species and PFJ antioxidant activity in treated HepG2 cells

Cell culture conditions and preparation of mitochondrial DNA were as previously reported [4]. HepG2 cells, a good model for studying the effects of drugs such as AZT on mtDNA [18], were cultured for thirty days in four separate conditions: (1) 7 μM AZT (Sigma, St. Louis,

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Received January 05, 2015; **Accepted** March 14, 2015; **Published** March 24, 2015

Citation: Osborne AE, Sanchez JA, Wangh LJ, Sambanthamurthi R, Hayes KC (2015) Oxidative Damage is not a Major Contributor to AZT-Induced Mitochondrial Mutations. J AIDS Clin Res 6: 441. doi:10.4172/2155-6113.1000441

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MO), (2) 25 µg gallic acid equivalents (GAE)/mL PFJ (a gift from the Malaysian Palm Oil Board), (3) 7 µM AZT and 25 µg GAE/mL PFJ, or (4) culture media alone. After thirty days of treatment, triplicate samples of 2×10^4 cells/mL were placed in a 96-well plate in the above conditions, and cells were allowed to adhere for twenty-four hours prior to staining for reactive species. To confirm the anti-oxidant activity of PFJ in HepG2 cells, a set of wells with untreated or PFJ-treated cells were also incubated for 60 minutes at 37°C in the presence or absence of 1mM H_2O_2, the main radical species generated by mitochondria. To preclude the possibility that PFJ directly inactivated H_2O_2 in the culture media, all extracellular traces of PFJ were removed by multiple washes before addition of H_2O_2. After incubation with H_2O_2, all wells were rinsed with PBS twice to remove H_2O_2. To stain for mitochondrial-specific reactive species (ROS and RNS), cells cultured in the four conditions above and H_2O_2 treated cells were incubated in serum-free Eagle's Minimum Essential Medium (EMEM, ATCC Manassas, VA) supplemented with 500nM MitoTracker® Orange CM-H2TMRos (Life Technologies, Grand Island, NY) for 15 min at 37°C [19]. After staining wells were again rinsed twice with PBS to remove unincorporated dye and read in an Infinite 200 PRO fluorescent plate reader (Tecan, Männedorf, Switzerland) at a 579 nm excitation wavelength and a 599 nm emission wavelength.

Analysis of AZT mutational spectrum

Mitochondrial DNA was isolated as previously described [20]. Briefly, 1000 cells were lysed in 14 µL of Quantilyse [21]. Samples were stored at -20°C. Mutational analysis and DNA sequencing were conducted as described previously [4].

Statistical analysis

Statistical analysis of the antioxidant activity of PFJ data was carried out using a one-way ANOVA test followed by a Tukey HSD test. The test was performed using 95% significance (p-value of less 0.05).

Results

Palm Fruit Juice has antioxidant activity against H_2O_2-induced. ROS in HepG2 cells

HepG2 cells were treated with H_2O_2 to test the anti-oxidant activity of PJF in our system since H_2O_2 is the major source of ROS in dysfunctional mitochondria [22]. Hydrogen peroxide in the absence of PFJ increased reactive species 24-fold. In contrast, PFJ alone did not affect ROS levels. Importantly, H_2O_2 added to cells grown in the presence of PFJ for thirty days failed to elicit an increase in ROS (Table 1). These results demonstrate that one or more components of PFJ are effective inhibitors of H_2O_2 induced-ROS in HepG2 cells and are consistent with the reported antioxidant activity of PFJ in chemical assays [16].

Palm fruit juice did not decrease overall reactive species levels in AZT-treated HepG2 cells

The capacity of PFJ to affect AZT-induced increases in reactive species was investigated. In agreement with a previous report [7], HepG2 cells treated with a mutagenic concentration of AZT (7 µM) for thirty days developed higher levels of reactive species compared to untreated cells (Table 1). Palm fruit juice treatment alone, which is not mutagenic [23], did not increase reactive species above background. Despite using a concentration of PFJ that mitigated mtDNA mutations [23], AZT-induced reactive species remained elevated in cells co-treated with AZT and PFJ (Table 1). These results uncouple PFJ mitigation of AZT-induced mtDNA damage from the ability of PFJ to mitigate reactive species generated by AZT.

The spectrum of AZT-induced mutations was inconsistent with oxidative damage

If AZT-induced mtDNA mutations resulted from direct oxidative damage, the mutations should exhibit a preponderance of G → T/C → A transversions [10,11]. Although AZT treatment for 30 days resulted in a wide spectrum of mutations, G → T/C → A transversions characteristic of oxidative DNA damage did not increase above the background observed in untreated cells (Figure 1). The only mutations associated with oxidative damage observed above background were G → C/C → G (20%, Figure 1). The most predominant mutations observed (G → A/C → T and T → C/A → G, 80% collectively) are characteristic of mtDNA polymerase errors [24,25]. These observations suggest that AZT-induced mutations were not likely the result of direct oxidative damage to mtDNA.

Discussion

A major conclusion from this work is that oxidative stress caused by AZT treatment is only a minor contributor to mtDNA mutations. The hypothesis that oxidative damage might be the major driver of AZT-induced mutations was based on observations that (1) AZT treatment induces the formation of reactive species [5-9]; (2) these reactive species cause oxidative DNA damage [10-14]; (3) oxidative DNA damage promotes the formation of characteristic G → T/C → A transversion mutations [10,11]; (4) PFJ has strong scavenging activity against hydrogen-peroxide-induced ROS *in vitro* [16]; and PFJ mitigates AZT-induced mutations [26]. This hypothesis predicts that PFJ mitigation of these mutations should be accompanied by a corresponding decrease in reactive species. However, direct measurements of overall reactive species in HepG2 cells co-treated with PFJ and AZT showed that mitigation of AZT-induced mutations occurred in the absence of a significant decrease in these reactive species, even though PFJ was demonstrated to have strong antioxidant activity against ROS.

The failure of H_2O_2 to induce ROS when cultured with PFJ could simply have been an artefact due to direct inactivation of H_2O_2 by PFJ in the media rather than inhibition of ROS production within the cells. To rule out this possibility, cells were cultured in the presence of PFJ, and all extracellular traces of PFJ were then removed by multiple washes before treating the cells with H_2O_2 (see Materials and Methods). Under these conditions PFJ still prevented formation of H_2O_2-induced ROS. These results are in agreement with the strong hydrogen peroxide

	HepG2 Treatment					
	Untreated	H_2O_2	PFJ	H_2O_2+PFJ	AZT	AZT+PFJ
Normalized Fluorescence	$0 \pm 3^{a,c}$	$1417 \pm 15^{a,b}$	$59 \pm 42^{b,c}$	46 ± 33^{b}	430 ± 170	$619 \pm 18^{a,c}$

This table shows the difference in normalized fluorescence of MitoTracker® Orange CM-H2TMRos dye between H_2O_2 and AZT treated cells. Palm fruit juice significantly lowers ROS in H_2O_2 samples while not lowering reactive species generated from AZT. Fluorescence was normalized by normalizing for the number of cells per well and then subtracting the background fluorescence of the untreated samples. The mean standard deviation is given. Similarly superscripted letters correspond to a significant difference ($p<0.05$) between treatments by one-way ANOVA followed by a Tukey HSD test.

Table 1: Effects of PFJ treatment on H_2O_2- and AZT-induced reactive species.

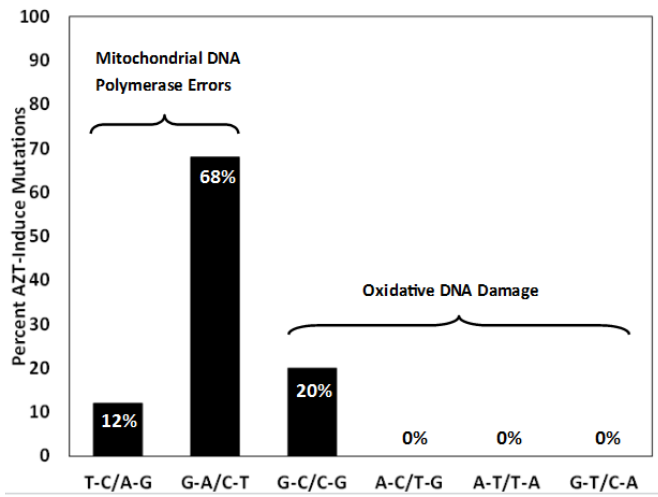

Figure 1: Treatment with 7 μM of AZT for 30 days did not generate the mutational spectra characteristic of oxidative damage as. The majority of mutations above background were those associated with mitochondrial DNA polymerase errors.

scavenging activity of PFJ *in vitro* [16] and indicate that PFJ inhibited ROS formation inside HepG2 cells.

Why, then, did PFJ not reduce the overall level of reactive species generated in AZT treated cells? Palm fruit juice may not counteract all reactive species the same way. Amatore et al. showed that reactive species induced by AZT treatment consists mostly of RNS (90%), with the remainder being of ROS (10%) [8]. The fluorescent dye used for detection of reactive species in this work (MitoTracker® Orange CM-H2TMRos) detects both ROS and RNS [27]. However, PFJ appears to fall into the category of antioxidants that act predominantly on ROS, and not RNS, unlike vitamin C and other antioxidants which act on both [12,28]. The fact that AZT treatment generates mostly RNS together with the selective anti-oxidant activity of PFJ against ROS, not RNS, might account for failure of PFJ to appreciably reduce the overall reactive species generated by AZT. Since oxidative damage to DNA by RNS generates the same type of mutations as ROS [11,12], and PFJ mitigates AZT mutations without altering the total AZT-generated reactive species, oxidative damage must not be a major contributor to AZT mutagenesis. Use of non-discriminating antioxidants, such as vitamin C [28], may have led to the incorrect conclusion that oxidative damage accounts for AZT mutagenesis. Other groups using next-generation sequencing and other methods of mutation detection have also recently questioned whether oxidative damage is the major contributor to mtDNA mutations in general [29-31].

The working hypothesis also predicted that AZT-induced mutations should exhibit a preponderance of G → T/C → A transversions, characteristic of oxidative damage by ROS and RNS [11,12]. However, such transversion mutations were not increased above background (Figure 1). These observations provide independent evidence against oxidative damage of mtDNA as the main cause of AZT-induced mutations. Similar conclusions were reached when analysing mutations caused by other less frequent types of oxidative damage (Figure 1).

Graziewicz et al. reported that oxidative DNA lesions block mtDNA replication *in vitro*, which may result in mtDNA depletion [32]. Depletion of damaged mtDNA could explain the scarcity of oxidative mutations in the AZT mutational spectra. However, previous publications showed that neither AZT treatment, nor PFJ co-treatment, altered mtDNA copy number [4,23]. Thus, selective loss of mutated mtDNA would not account for the relative lack of signature mutations for oxidative damage in these experiments.

Given the present results, the working hypothesis that AZT-induced oxidative stress was the main cause of mtDNA mutations, must be re-examined. It is unambiguous that AZT causes oxidative stress: AZT treatment induces cellular and mitochondria-specific hydrogen peroxide, peroxynitrite and ROS, increases mitochondria lipid peroxidation, and increases oxidation of mitochondrial glutathione *in vivo* and in primary and established cell lines [1-3,6-8]. It is also clear that AZT treatment causes the accumulation of 8-oxo-dG (up to 38% of all deoxyguanosine residues in samples treated with sub-optimal doses of AZT for 30 days) [33,34]. Why then were there not more mutations due to oxidative damage? Four explanations are plausible. First, the magnitude of the published 8-oxo-dG measurements, an index of oxidative DNA damage, may have been overestimated since the original studies did not distinguish between oxidation of free deoxynucleotides and oxidation of deoxynucleotides in mtDNA [35], nor did they control for artifactual oxidation during mtDNA isolation [36]. Second, even if 8-oxo-dG occurs it is weakly mutagenic; i.e., 8-oxo-dG has mutation frequencies of only 2.5-4.8% in nuclear DNA [35]. Third, the mutagenic effect of 8-oxo-dG and 8-nitro-dG on mtDNA may even be less than predicted, since different polymerases respond differently to different lesions. *In vitro* studies with purified enzymes showed that mitochondrial DNA polymerase gamma inserts dideoxyadenosine opposite 8-oxo-dG about 10% of the time [37]. Fourth, oxidative DNA lesions are efficiently repaired by redundant base excision repair and nucleotide excision repair [35,38].

In light of this body of knowledge and the findings reported here a new working hypothesis to explain the mutagenic effects of AZT emerges. Although oxidative damage plays a role in AZT toxicity, our results indicate that oxidative stress is likely a minor contributor to AZT-induced mutations. How might PFJ be mitigating AZT-induced mutagenesis? One possibility is that AZT-induced mutations may result from changes in the fidelity of the mitochondrial polymerase gamma [39]. This hypothesis is consistent with our observation that G → A/C → T mutations characteristic of mitochondrial DNA polymerase errors [23-25] predominated among AZT mutations. Accordingly, PFJ might interact with the polymerase to preserve its fidelity. Alternatively, AZT might also alter the cellular nucleotide pools leading to mitochondrial DNA polymerase errors [40,41]. Palm fruit juice may be preventing these imbalances. Current research is focusing on evaluating the possible mechanism of action of palm fruit juice.

Acknowledgements

This work was funded 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.

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