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Assessment of genomic instability induced by nucleoside reverse transcriptase inhibitors

Avaliação da instabilidade genômica induzida por inibidores da transcriptase reversa análogos de nucleosídeos

Evaluación de la inestabilidad genómica inducida por inhibidores de la transcriptasa reversa análogos de nucleósidos

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Abstract
Antiretroviral therapy (ART) has greatly enhanced the quality of life and life expectancy of people living with HIV. However, concerns about the genotoxic potential of nucleoside reverse transcriptase inhibitors (NRTIs), particularly zidovudine (AZT) and lamivudine (3TC), remain relevant. This study aimed to evaluate the genotoxic effects of AZT, 3TC, and their combination (2:1 ratio) in CHO-K1 cells using the alkaline comet assay. A quantitative laboratory research was carried out. Cells were exposed to increasing concentrations of each drug individually and in combination for 24 hours. DNA damage was quantified as the percentage of DNA in the comet tail. AZT significantly increased DNA damage at the two highest concentrations (900 and 1350 µM), while 3TC did not induce genotoxic effects at any concentration tested. The AZT+3TC combination caused significant DNA damage at the two highest doses compared to the negative control, with no evidence of synergistic interaction relative to AZT alone. These findings align with previous studies and highlight the importance of evaluating DNA integrity in ART regimens. The comet assay proved to be a sensitive tool for detecting NRTI-induced genotoxicity, and the data underscore the need for continued monitoring of combination therapies to ensure long-term treatment safety.
Keywords: Genotoxicity; Comet assay; AZT; 3TC.

Resumo
A terapia antirretroviral (TARV) tem melhorado significativamente a qualidade e a expectativa de vida de pessoas vivendo com HIV. No entanto, persistem preocupações quanto ao potencial genotóxico dos inibidores da transcriptase reversa análogos de nucleosídeos (ITRNs), em especial a zidovudina (AZT) e a lamivudina (3TC). Este estudo teve como objetivo avaliar os efeitos genotóxicos do AZT, do 3TC e da combinação de ambos (proporção 2:1) em células CHO-K1, utilizando o ensaio cometa em sua versão alcalina. Uma pesquisa laboratorial quantitativa foi realizada. As células foram expostas a concentrações crescentes de cada fármaco, isoladamente e em combinação, por 24 horas. Os danos ao DNA foram quantificados como a porcentagem de DNA presente na cauda do cometa. O AZT induziu aumento significativo de danos ao DNA nas duas maiores concentrações testadas (900 e 1350 µM), enquanto o 3TC não apresentou efeitos genotóxicos em nenhuma das concentrações avaliadas. A combinação AZT+3TC causou danos significativos ao DNA nas duas maiores doses em comparação ao controle negativo, sem evidências de interação sinérgica em relação ao AZT isolado. Esses achados reforçam a importância da avaliação da integridade do DNA nos

esquemas de TARV. O ensaio cometa demonstrou ser uma ferramenta sensível para detectar genotoxicidade induzida por ITRNs, evidenciando a necessidade de monitoramento contínuo das terapias combinadas.
Palavras-chave: Genotoxicidade; Ensaio cometa; AZT; 3TC.

Resumen
La terapia antirretroviral (TAR) ha mejorado significativamente la calidad y la expectativa de vida de las personas que viven con VIH. Sin embargo, persisten preocupaciones sobre el potencial genotóxico de los inhibidores de la transcriptasa reversa análogos de nucleósidos (ITRNs), especialmente la zidovudina (AZT) y la lamivudina (3TC). Este estudio tuvo como objetivo evaluar los efectos genotóxicos del AZT, del 3TC y de la combinación de ambos (proporción 2:1) en células CHO-K1, utilizando el ensayo cometa en su versión alcalina. Se realizó una investigación cuantitativa de laboratorio. Las células fueron expuestas a concentraciones crecientes de cada fármaco, de forma individual y combinada, durante 24 horas. El daño al ADN se cuantificó como el porcentaje de ADN presente en la cola del cometa. El AZT indujo un aumento significativo del daño al ADN en las dos concentraciones más altas evaluadas (900 y 1350 µM), mientras que el 3TC no mostró efectos genotóxicos en ninguna de las concentraciones probadas. La combinación AZT+3TC causó daños significativos al ADN en las dos dosis más altas en comparación con el control negativo, sin evidencia de interacción sinérgica en relación con el AZT aislado. Estos hallazgos refuerzan la importancia de evaluar la integridad del ADN en los esquemas de TAR. El ensayo cometa demostró ser una herramienta sensible para detectar la genotoxicidad inducida por ITRNs, destacando la necesidad de un monitoreo continuo de las terapias combinadas.
Palabras clave: Genotoxicidad; Ensayo cometa; AZT; 3TC.

1. Introduction

Human immunodeficiency virus (HIV) infection continues to represent a major global health concern, demanding sustained attention from both the scientific and medical communities. Despite notable advancements in diagnosis, treatment, and prevention strategies, the infection remains incurable and is associated with significant morbidity and mortality. The introduction and widespread use of antiretroviral therapy (ART) have transformed the clinical course of HIV, improving life expectancy and quality of life for millions of individuals. However, long-term therapeutic use raises critical questions about drug safety and the potential for adverse effects, particularly at the genetic and cellular levels (UNAIDS, 2023; Bbosa et al., 2024).

Nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine (AZT) and lamivudine (3TC), are integral components of first-line ART regimens. These agents function by mimicking natural nucleosides, incorporating into viral DNA during replication, and causing premature chain termination (Suan et al., 2024). Their combined use has demonstrated synergistic antiviral effects, contributing to their widespread adoption in clinical practice (De Clercq, 2009).

However, concerns have been raised regarding the potential genotoxic effects of NRTIs. AZT, in particular, has been associated with mitochondrial toxicity due to its inhibition of mitochondrial DNA polymerase γ, leading to mitochondrial DNA depletion and increased oxidative stress (Holec et al., 2017; Gardner et al., 2013). While 3TC exhibits a comparatively lower toxicity profile (Maagaard and Kvale, 2009), the combined administration of these drugs may exacerbate genotoxic risks, necessitating comprehensive evaluations of their safety profiles.

The alkaline comet assay is a sensitive and reliable method for detecting DNA strand breaks and alkali-labile sites at the single-cell level. Utilizing this assay in Chinese hamster ovary (CHO-K1) cells provides a robust platform for assessing the genotoxic potential of pharmaceutical compounds, aligning with international guidelines for genetic toxicology testing (Collins et al., 2023; OECD, 2018). CHO-K1 cells, in particular, are recommended due to their sensitivity to DNA-damaging agents (Shardosim et al., 2022; Trintinaglia et al., 2025).

In this context, the present study aims to investigate the genomic instability induced by AZT and 3TC, both individually and in combination at a 2:1 ratio, using the alkaline comet assay in CHO-K1 cells. By quantifying DNA damage under these treatment conditions, the research seeks to elucidate the genotoxic implications of NRTI-based therapies, thereby informing safer and more effective HIV treatment protocols.

2. Methodology

A quantitative laboratory research was carried out (Pereira et al., 2018) using simple descriptive statistics with mean values, standard deviation, absolute frequency and relative percentage frequency (Shitsuka et al., 2014) and with statistical criteria (Vieira, 2021).

Chemical agents

AZT (3′-azido-3′-deoxythymidine; CAS: 30516-87-1) and 3TC (2′-deoxy-3′-thiacytidine; CAS: 13678-17-4) were obtained from the Goiás Chemical Industry (IQUEGO, GO, Brazil) and the Tropical Diseases Hospital (HDT, Goiânia, GO, Brazil). Solutions and dilutions were freshly prepared immediately before use. AZT, 3TC, and the AZT+3TC combination were dissolved in Dulbecco's Modified Eagle's Medium (DMEM).

Cell culture and drug treatment

CHO-K1 cells were obtained from the Rio de Janeiro Cell Bank (BCRJ, number 0069). They were cultured as monolayers in 75 cm² plastic culture flasks containing DMEM (Gibco, São Paulo, SP, Brazil), supplemented with fetal bovine serum (Gibco, São Paulo, SP, Brazil) and 1% penicillin-streptomycin, and maintained at 37°C in a 5% CO_2 incubator.

NRTI concentrations were defined based on cell viability assays. Cells were exposed to different NRTI concentrations for 24 hours. After incubation, cells were trypsinized using trypsin/EDTA (Gibco, São Paulo, SP, Brazil), and 10 µL of the cell suspension was mixed with 10 µL of trypan blue and counted in a hemocytometer. Only concentrations that resulted in more than 70% viable cells (data not shown) were selected for testing. The following concentrations were used: AZT (1350 µM, 900 µM, 600 µM, 300 µM), 3TC (675 µM, 450 µM, 300 µM, 150 µM), and the AZT + 3TC combination (1350 µM + 675 µM, 900 µM + 450 µM, 600 µM + 300 µM, 300 µM + 150 µM).

To evaluate genotoxicity induced by the individual and combined treatments, each protocol was performed in triplicate on different days to ensure reproducibility. Positive (EMS 500 µM) and negative (DMEM) controls were included in all experiments. For each assay, 1 x 10^5 CHO-K1 cells were seeded in complete DMEM in 24-well plates and incubated for 24 h at 37°C under 5% CO_2. After this period, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and treated with the selected concentrations of NRTIs for 24 h in serum-free medium.

The AZT + 3TC combination was prepared at a 2:1 ratio, based on protocols used in clinical trials for HIV patients (De Clercq, 2009). In parallel with each combined treatment, the two drugs were also administered individually to separate groups of cells at the same concentrations used in the combination.

Comet assay

The comet assay was performed according to Tice et al. (2000), with modifications. Microscope slides were pre-coated with 1.5% normal melting point agarose (CAS: 9012-36-6, Invitrogen). Then, 40 µL of the cell suspension was mixed with 160 µL of 0.5% low melting point agarose (CAS: 9012-36-6, Invitrogen) at 37°C and layered onto the prepared slides. Coverslips were placed over the gel and solidified at 4°C for 15 minutes. After solidification, coverslips were removed, and slides were immersed in lysis solution (89 mL stock solution; 10 mL DMSO; 1 mL Triton X-100; pH 10.0) at 4°C for at least 1 hour, protected from light.

Following lysis, slides were washed in neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 minutes and then subjected to electrophoresis in a horizontal tank, immersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 20 minutes. Electrophoresis was performed at 36 V and 300 mA for 20 minutes. After electrophoresis, slides were washed again with neutralization buffer for 15 minutes and fixed in 100% ethanol. Slides were stained with ethidium bromide and analyzed

using fluorescence microscopy (Olympus BX41 – excitation filter 515–560 nm; barrier filter 590 nm). Image analysis was performed using the Comet Assay IV software (Perceptive Instruments, UK).

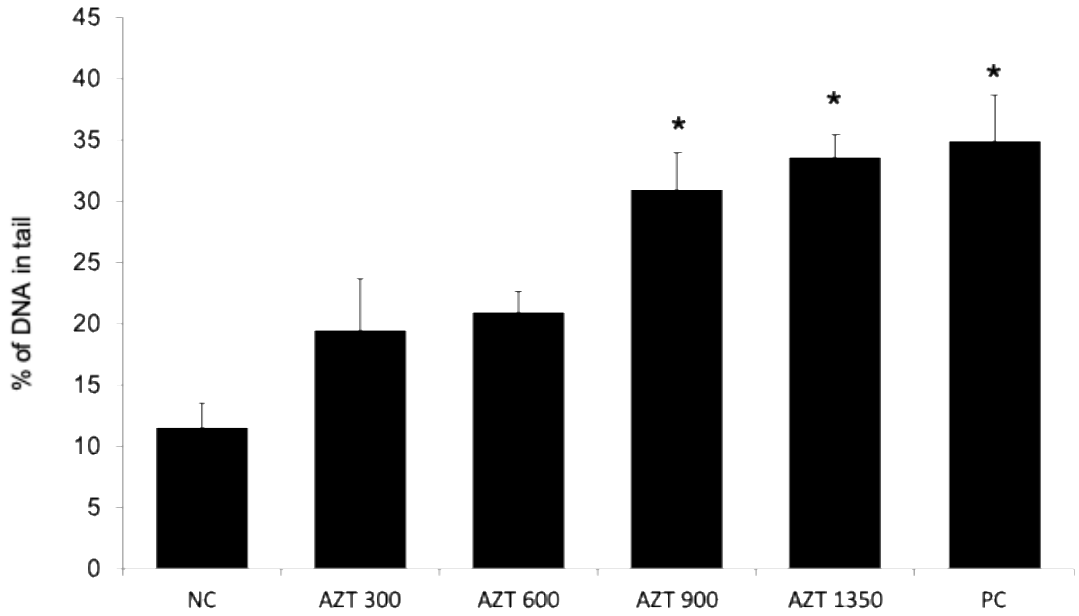
Statistical analysis

Statistical analyses were performed using SPSS 22 software. Data were analyzed using ANOVA followed by Dunnett's post hoc test. Statistical significance was determined by comparing negative controls with treated groups, both for individual and combined treatments. A p-value < 0.05 was considered statistically significant.

3. Results

The genotoxic potential of AZT, 3TC, and the AZT/3TC combination in CHO-K1 cells was evaluated using the comet assay. Data regarding the effects of AZT alone are shown in Figure 1. The results demonstrated that the two highest concentrations of AZT (900 µM and 1350 µM) induced significant increases in DNA damage, as evidenced by a higher percentage of DNA in the comet tail, compared to the negative control.

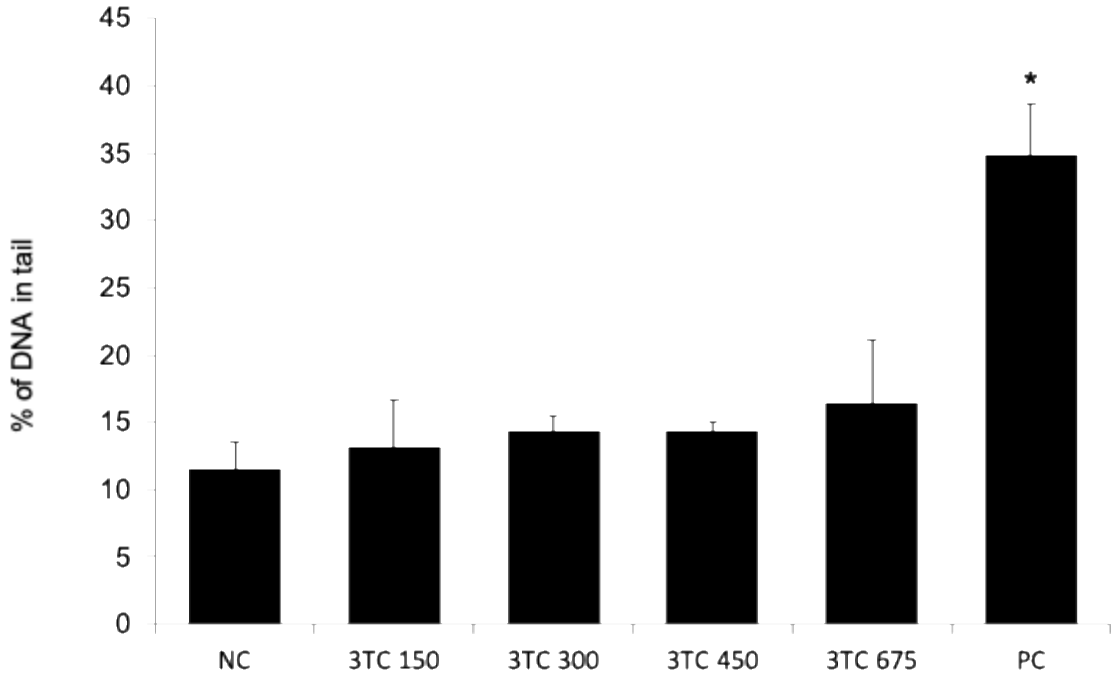
Figure 1 - DNA damage in CHO-K1 cells after exposure to different concentrations of AZT (300 – 1350 µM). The percentage of DNA in the comet tail is expressed as mean ± standard deviation (three independent experiments performed in duplicate). Negative control (NC) – DMEM culture medium. Positive control (PC) – EMS at 0.5 mM. *P < 0.05.



Source: Authors.

The genotoxic effect of 3TC was also assessed, and the results are presented in Figure 2. No significant differences were observed between the various 3TC treatments and the negative control. The only positive result was observed in cells exposed to EMS, the assay's positive control.

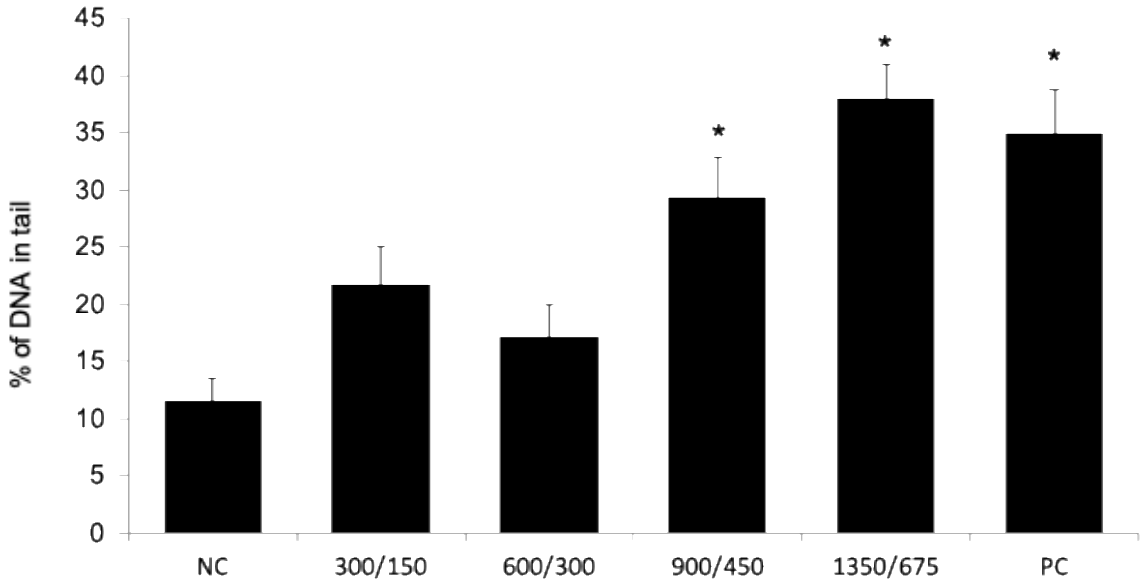
Figure 2 - DNA damage in CHO-K1 cells after exposure to different concentrations of 3TC (150 – 675 µM). The percentage of DNA in the comet tail is expressed as mean ± standard deviation (three independent experiments performed in duplicate). Negative control (NC) – DMEM culture medium. Positive control (PC) – EMS at 0.5 mM. *P < 0.05.



Source: Authors.

Following the evaluation of the isolated drugs, the AZT+3TC combination was tested at four concentrations (1350 µM + 675 µM, 900 µM + 450 µM, 600 µM + 300 µM, and 300 µM + 150 µM). The results of this evaluation are shown in Figure 3. Cells exposed to the AZT+3TC combinations at 900 µM + 450 µM and 1350 µM + 675 µM exhibited a significant increase in DNA damage compared to the negative control. No statistically significant differences were observed for the combinations at 300 µM + 150 µM and 600 µM + 300 µM.

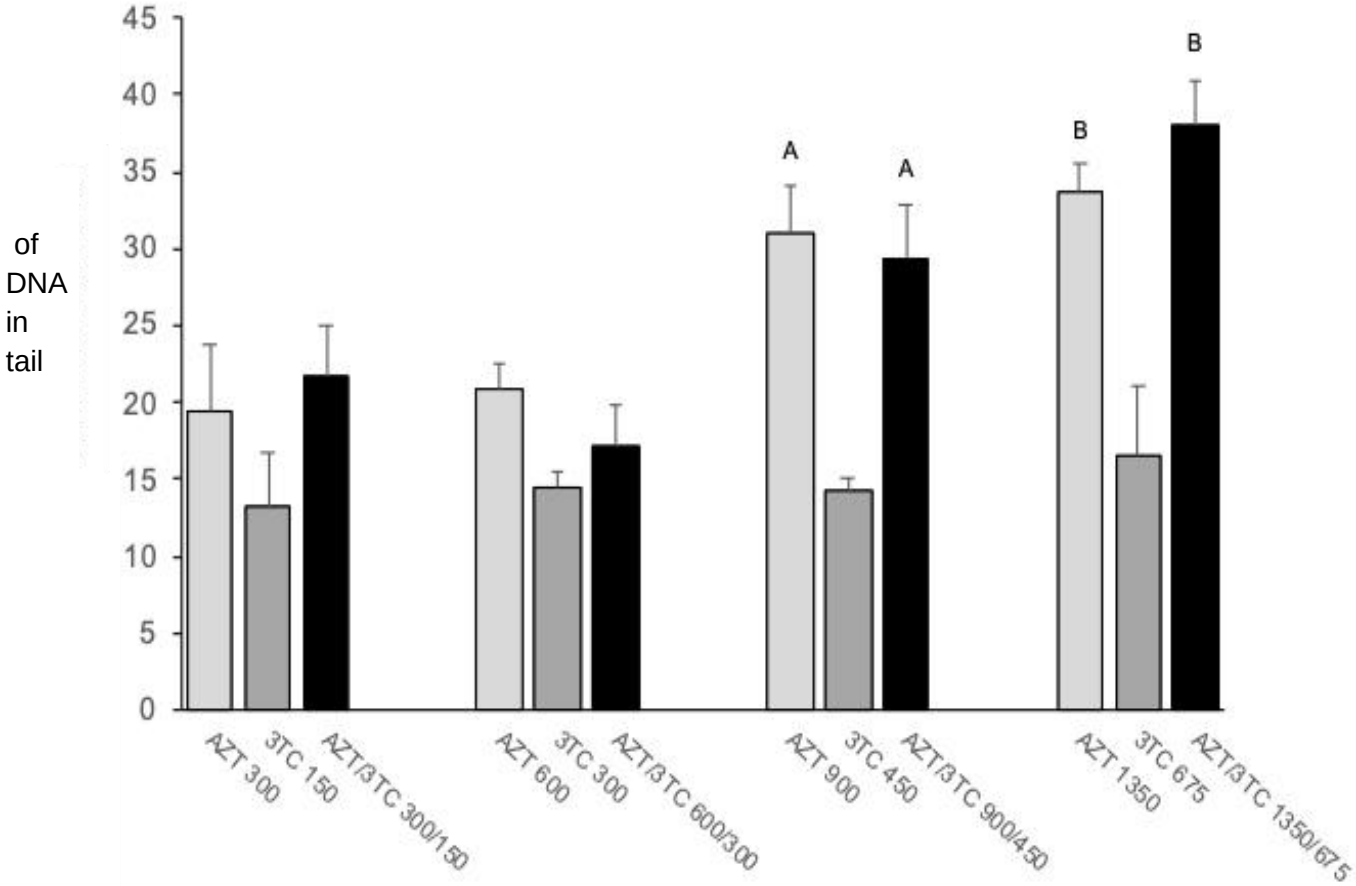
Figure 3 - DNA damage in CHO-K1 cells after exposure to different concentrations of the AZT/3TC combination. The percentage of DNA in the comet tail is expressed as mean ± standard deviation (three independent experiments performed in duplicate). Negative control (NC) – DMEM culture medium. Positive control (PC) – EMS at 0.5 mM. *P < 0.05.



Source: Authors.

Figure 4 presents the statistical comparison between the combined treatments and the corresponding individual drugs. The AZT + 3TC combination at 1350 µM + 675 µM and 900 µM + 450 µM showed significantly greater genotoxicity than 3TC alone at 675 µM and 450 µM, respectively.

Figure 4 - Comparison of DNA damage frequency between isolated and combined drug treatments at the same tested concentrations. The percentage of DNA in the comet tail is expressed as mean ± standard deviation (three independent experiments performed in duplicate). Negative control (NC) – DMEM culture medium. Positive control (PC) – EMS at 0.5 mM. A: Significantly different ($P < 0.001$) compared to the 3TC-alone group (450 µM). B: Significantly different ($P < 0.001$) compared to the 3TC-alone group (675 µM).



Source: Authors.

4. Discussion

In this study, we analyzed the ability of the NRTIs AZT and 3TC to induce DNA damage in CHO-K1 cell cultures using the comet assay. CHO-K1 cells are commonly used in genetic toxicology studies and are among the cell lines recommended for detecting compounds that act directly on DNA (Trintinaglia et al., 2025). The comet assay is a widely established and highly sensitive genotoxicity test employed to detect a broad spectrum of DNA damage (Tice et al., 2000; Calabrese and Selby, 2024). In its alkaline version, this assay detects DNA strand breaks and alkali-labile sites, with the extent of DNA migration reflecting the degree of cellular DNA damage (Collins et al., 2023). In our experimental results, CHO-K1 cells exposed for 24 hours to individual NRTIs showed genotoxic activity only for AZT at the two highest concentrations (900 and 1350 µM). Cells treated with 3TC did not show increased DNA damage compared to the negative control at any of the concentrations tested.

Escobar et al. (2007) conducted a series of *in vitro* and *in vivo* experiments to investigate the genotoxicity of AZT-based treatments. In human H9 lymphoblastoid cell cultures exposed to AZT for 24 hours, the comet assay revealed an increased DNA damage when electrophoresis was performed at pH 13 at concentrations of 0.2, 0.4, 0.8, and 1.2 µM. Similarly, Tripathi et al. (2008) used the comet assay to assess the transplacental genotoxicity of AZT in mice. The compound was administered from gestational days 16 to 20, and the assay was performed on lymphocytes, bone marrow, liver, and kidney cells from newborn pups, where significant DNA damage was observed in all tissues analyzed. No studies were found reporting comet assay-detectable DNA damage in cells treated with 3TC.

Grando et al. (2020) evaluated the ability of AZT and 3TC to induce complex genomic alterations using the cytokinesis-block micronucleus (CBMN) assay in Chinese hamster CHO-K1 cells. A 24-hour treatment with individual NRTIs showed that AZT increased the frequency of micronuclei and nucleoplasmic bridges. No significant changes were observed in any parameters following 3TC exposure in CHO-K1 cells. On the other hand, Lourenço et al. (2010) assessed the aneugenic and clastogenic effects of AZT, 3TC, and d4T using the CBMN assay in cultured human lymphocytes. The results showed that AZT and 3TC induced micronuclei formation only in binucleated cells, while d4T induced micronuclei in both mononucleated and binucleated cells. The authors concluded that AZT and 3TC caused chromosomal damage consistent with clastogenic activity, whereas d4T induced both clastogenic and aneugenic effects.

Both AZT and dideoxynucleosides, as chain terminators, may cause irreversible DNA damage, preventing repair via excision or post-replication mechanisms, even in cells with competent repair systems (Mamber et al., 1990). In addition, AZT photodegradation products can induce the formation of 8-oxoG *in vitro* (Hashiguchi et al., 2004), leading to DNA modifications such as DNA strand breaks, DNA-protein cross-links, and base oxidation (Wu et al., 2013). These mechanisms may explain the increased percentage of DNA in the tail observed in our experiments, especially at higher AZT concentrations, as these types of damage are detectable by the comet assay.

Potential strategies to mitigate the additive or synergistic cytotoxic and mutagenic effects of NRTI combinations are limited and may include the use of less toxic drug combinations, the addition of cytoprotective or antimutagenic agents to current drug cocktails, or the development of alternative antiviral therapies with reduced toxicity (Tompa et al., 2021; Brochard et al., 2023). Indeed, ART typically involves combining two or more NRTIs with another class of antiretroviral to slow the emergence of HIV mutations, which may lead to viral resistance and treatment failure when using single-drug regimens (Tao et al., 2024). In this context, assessing the genotoxic effects of NRTI combinations is essential to evaluate the risks associated with co-exposure to these drugs.

Our results showed that exposure to two NRTIs (AZT + 3TC) was genotoxic to CHO-K1 cells at the highest concentrations tested. Additionally, significant differences were observed when the combination treatment was compared to 3TC alone. Similar results were reported by Grando et al. (2020), using the cytokinesis-block micronucleus assay (CBMN). In that study, the authors found that the AZT + 3TC combination significantly increased the frequency of micronuclei. The analysis of drug interactions suggested antagonistic effects across all AZT + 3TC concentrations. These findings highlight the importance of investigating the genotoxic profiles of NRTIs to develop safer intervention strategies for antiretroviral treatment protocols.

The triphosphate metabolites, active components of all NRTIs, play a key role in inhibiting human mitochondrial DNA polymerase γ (pol γ), with varying degrees of efficacy. The phosphorylation reactions that activate NRTIs occur intracellularly and depend on the action of several cellular kinases (Bazzoli et al., 2010). The simultaneous presence of two or more analogs within cells can disrupt the balance of the deoxynucleotide triphosphate (dNTP) pool, potentially leading to genomic instability and increasing the risk of both nuclear and mitochondrial DNA damage—whether repairable or permanent

(Wheeler and Mathews, 2011; Chen et al., 2016). In one study, Chen and Oshana (1987) investigated reverse transcriptase inhibition using dideoxynucleoside triphosphates and established the following inhibitory efficacy ranking: ddTTP > ddGTP > ddCTP > ddATP. These antimetabolites interfere with various enzymes essential for DNA synthesis or induce subtle alterations in DNA bases (Painter et al., 2004; Fernandes et al., 2023). Nonetheless, the observed increase in DNA damage in our experiments—particularly at the higher concentrations of the AZT + 3TC combination—can most likely be attributed to AZT, given its genotoxic potential in isolated treatment.

5. Conclusion

The use of different bioassays that detect multiple genetic endpoints is essential for the proper characterization of the genotoxic profile of various classes of antiretroviral drugs. In this study, the comet assay was employed to evaluate the genetic toxicity of AZT, 3TC, and the AZT+3TC combination. Overall, the results obtained are consistent with previously published scientific literature on AZT and 3TC. It was observed that AZT exhibits genotoxicity at high concentrations, whereas 3TC, in most tests, does not cause DNA damage in cells.

Antiretroviral therapy (ART) is an indispensable tool in controlling HIV infection, allowing many individuals to live long and healthy lives. By combining antiretroviral drugs, it is possible to suppress viral load, delay disease progression, and prevent transmission. However, to maximize these benefits, ART must be carefully managed and individualized, ensuring treatment adherence while minimizing adverse effects. The results of this study indicated a genotoxic effect associated with the treatment of cells with the AZT+3TC combination. However, no synergistic or potentiated effect was observed when compared to AZT alone. Indeed, future studies should focus on evaluating the genotoxicity of different antiretroviral combinations, particularly involving drugs from different classes, based on currently available therapies.

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