

GENOMIC INSTABILITY AND CENTROSOMAL AMPLIFICATION INDUCED BY THE ANTIRETROVIRAL AGENT AZT.

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Abstract

Materials & Methods

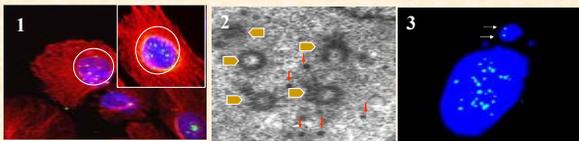
Results

- Patients infected with HIV-1 undergo therapy with antiretroviral nucleoside reverse-transcriptase inhibitors, among which AZT is used frequently.
- In cultured cells, exposure to the carcinogenic agent AZT induces cell cycle arrest, micronuclei, sister chromatid exchanges, shortened telomeres, and alterations in gene expression. A predicted consequence of these events is genomic instability.
- In this study two normal human mammary epithelial cell (NHMEC) strains were exposed to AZT for 24 hours and analyzed for aneuploidy by immunofluorescence and confocal microscopy. Centrosomal amplification, identified by a pericentrin antibody and evidenced by the percentage of cells containing ≥ 2 centrosomes, was evaluated. The NHMEC strain 05, previously identified as a strain that incorporates high levels of AZT into DNA, showed centrosomal amplification in 31.67% of cells exposed to 200 μ M AZT for 24 hrs and 5.83% centrosomal amplification in unexposed cells. The AZT strain NHMEC 40, which did not incorporate AZT into DNA, showed centrosomal amplification in 20.0% of cells exposed for 24 hrs to 200 μ M AZT and 7.83% centrosomal amplification in unexposed cells.
- Because aberrations in centrosome morphology are associated with chromosomal mis-segregation, micronuclei bearing intact chromosomes, stained using a CREST antibody, were scored revealing increased positive signal for centromeric kinetochores within micronuclei of cells exposed to AZT. The NHMEC 05 strain exposed to 200 μ M AZT for 24 hrs showed 36.4% of micronuclei containing centromeric signals compared to 25.9% of the control, respectively.
- Immunohistochemistry with an Aurora A antibody revealed abnormal polarity in the mitotic spindle. Western blot studies confirmed an up-regulation of the protein in NHMECs 05. Flow cytometric studies revealed that cells bearing multiple centrosomes are able to replicate and exhibit a cell cycle similar to cells bearing a normal number of centrosomes, thus producing daughter cells bearing an unbalanced number of chromosomes.
- Since centromeric positive micronuclei contribute to unbalanced chromosomal segregation or aneuploidy, the evidence presented here suggests that AZT induced genomic instability could be underlying the mechanism of AZT carcinogenicity.

Background

Exposure of Chinese Hamster Ovary (CHO) cells to AZT revealed:

- Striking disorganization of centrosomal material in the form of nuclear amplification and fragmentation in found by confocal microscopy (Photograph 1, circle) and confirmed by EM (Photograph 2, brown arrows)
- Pericentriolar bodies associated with the centrosome, absent in untreated cells (Photograph 2 red arrows)
- Kinetochores-positive micronuclei with centromeric portions of chromosomes lost from the nucleus and present in micronuclei (Photograph 3, arrows)



CHO cells exposed 24 hr to 200 and 400 μ M AZT

Objective

To explore the ability of AZT to induce centrosomal damage in normal human mammary epithelial cells (NHMECs).

Two strains of Normal Human Mammary Epithelial Cells (NHMEC) were exposed to 0, 10 or 200 μ M AZT for 24 hours.

The NHMEC strains were chosen based upon their AZT-DNA incorporation profile: **NHMEC 05** cells incorporate AZT into DNA, and **NHMEC 40** cells do not incorporate AZT into DNA

- Both, human cell strains, were analyzed by confocal microscopy, following staining with pericentrin antibody and a secondary antibody Alexa 488-conjugated (green), to identify centrosomes, and DAPI to localize nuclei.
- Cells were also stained with anti α -tubulin and a secondary antibody rhodamine-conjugated (red) to localize tubulin (Peloponnesse Jr., J. et al, PNAS 2005).
- Quantitation of positive pericentrin-Alexa 488 (centrosomal) signals was performed for 500-1000 cells.
- Identification of micronuclei containing positive kinetochores (centrosomal) signals was achieved utilizing a CREST antibody and a secondary antibody Alexa 488-conjugated and DAPI stain (blue), and counted in 25 micronuclei (50-1000 cells) from each treatment group.
- Aurora A identification was carried out with specific antibodies and a secondary antibody Alexa 488-conjugated (green).

Results

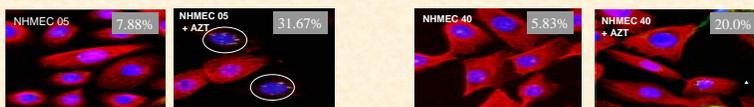


Figure 1 (A): Centrosomal amplification (% on right upper corner) plus loss of tubulin signal is seen in AZT-exposed NHMEC 05. Co-localization of tubulin and pericentrin (yellow spots) is also observed in NHMEC 05 (circles).

What percentage of the cells are abnormal?



Figure 1 (B): Expression of β tubulin (stained red) was lacking in 23.2 % NHMEC 05 cells, with an apparent lack of protein (green stain). Unexposed NHMEC 05 cells and NHMEC 40 cells had ~ 3.5 % of cells without staining for tubulin.

Are cells deprived of tubulin or just unable to polymerize tubulin?



Figure 2 (A): In a typical staining procedure, permeabilization is performed to eliminate the presence of "soluble" or un-polymerized tubulin

Figure 2 (B): In absence of permeabilization the unpolymerized tubulin remains as a homogeneous staining

Are cells containing multiple centrosomes able to cycle?

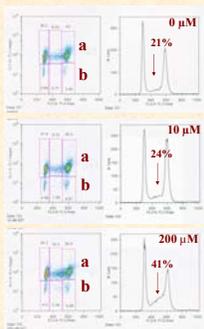


Figure 3: (Left) Both tubulin positive (a) and tubulin negative (b) cells cycle in a similar fashion in the control and the treated cultures. (Right): arrows indicate % S-phase cells. A clear increase in the amount of cells in S-phase is seen in the bottom panel (AZT 200 μ M).

These result indicate that cells deprived of tubulin are able to cycle in similar fashion to the tubulin positive cells.

Is there any evidence of abnormal distribution of chromosomes?

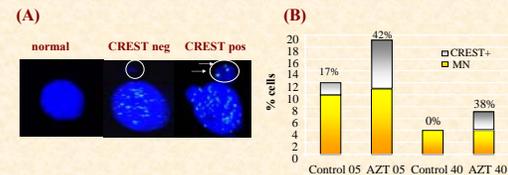
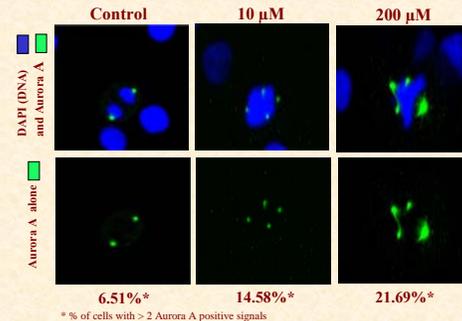


Figure 4 (A): Chromosomal loss, evidenced by kinetochores (CREST) positive micronuclei (arrows), contributes to aneuploidy, in NHMEC 05 exposed to 200 μ M AZT. (B): percentages of CREST positive micronuclei

Are there other manifestations of aneuploidy?



* % of cells with > 2 Aurora A positive signals

Figure 5: Amplification of the centrosomal activator, Aurora A in AZT exposed NHMEC 05 generates multipolar spindles

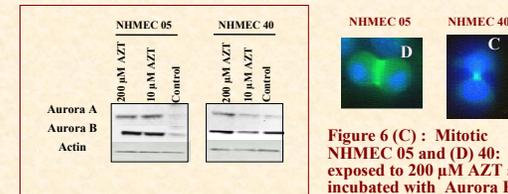


Figure 6 (B): Western blot analysis confirmed up-regulation of Aurora A and revealed up-regulation of Aurora B in NHMEC 05

Figure 6 (C): Mitotic NHMEC 05 and (D) 40: exposed to 200 μ M AZT and incubated with Aurora B antibody and Alexa 488 (green)

Summary

Exposure of NHMECs to AZT induced multiple manifestations of chromosomal instability including:

- Centrosomal amplification
- Lack of tubulin polymerization in cells bearing centrosomal amplification
- CREST positive micronuclei containing centromeric proteins
- Increase in centrosomal kinase activators Aurora A and B
- Multipolar metaphases

Conclusion

Genomic instability may be a major mechanism contributing to the carcinogenic potency of AZT.