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Safety profile of azt derivatives: phenylseleno moieties confer different cytotoxic responses in fresh human erythrocytes during *in vitro* exposures

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Abstract. The cellular effects of three molecules based on antiretroviral zidovudine (AZT) on erythrocytes viability and functionality was proposed and investigated in this *in vitro* study. A short-term treatment was conducted to estimate the effects of AZT and selenium-containing derivatives (10-500µM) on membrane damage, redox disturbance and thiol containing enzymes activity in freshly human erythrocytes. The effects of derivatives towards erythrocytes differed considerably. Overall, derivative 3 (methylphenylseleno) had similar patterns to prototypal AZT, showing no significant signals of cytotoxicity. The insertion of either chlorophenylseleno or, in a certain way, phenylseleno portions in the structure of AZT molecule was detrimental to erythrocytes and this effect seems to involve a pro-oxidant activity and disruption in thiol balance. This was not true for the derivative encompassing methylphenylseleno portion, making it a promising candidate for *in vivo* studies.

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Introduction: Some classes of nucleosides and nucleotides analogues have presented widespread utility as both antiviral and anticancer therapeutics (Galmarini et al. 2002; Kukhanova, 2012). One of the most studied is the thymidine deoxynucleoside analogue azido 3'-deoxythymidine or zidovudine (AZT). By suppressing HIV reverse transcriptase activity, AZT is often incorporated as one of the anti-retroviral drugs of choice in a combination therapy extremely effective in halting the HIV replication and slowing the progression of AIDS (Khandazhinskaya and Shirokova, 2013). Additionally, AZT has proven to be efficient against some types of cancer by

inhibiting the telomerase enzyme and cell cycle arrest (Abdulkarim and Bourhis, 2001; Gomez et al., 2012). Nevertheless, the pharmacological use of AZT in modern therapy has been limited because of its moderate toxicity, commonly manifested by anemia, neutropenia, and thrombocytopenia in patients in a long-term treatment (Brogan et al., 1987; Brogan and Zell, 1990).

Due to the current undesirable effects of the commercially anti-HIV and anticancer drugs, the development of new nucleoside molecules, including those based on AZT scaffold, has become an increasingly area of research. Employing different structural strategies, several research groups have synthesized and demonstrated the efficacy from a variety of AZT derivatives (Solyev et al., 2012; Shastina et al., 2013; Wang et al., 2014; Vasilyeva, and Bioorg, 2015; see the review from Khandazhinskaya et al., 2010). In this sense, Souza and collaborators (2015) showed the anti-tumoral activity of a series of AZT derivatives containing organoselenium moieties, on cultures of bladder carcinoma cells. Interestingly, selenium incorporation conferred to the molecules a notable thiol-like activity and improved the anti-tumor activity by activating the pro-apoptotic cell machinery. However, investigations toward healthy cells are still necessary to evaluate the safety of these derivatives. Regarding the hematological disturbances in patients treated with AZT, we asked whether erythrocytes could be affected by a short-term exposure to three AZT derivatives. To answer that, we addressed the erythrocyte hemolytic and oxidative potential as well as the reactivity on endogenous thiol and sulfhydryl-containing enzymes, all considered molecular targets for organochalcogens toxicity.

Methods: Synthesis of selenium compounds: The synthetized derivatives possessed additional phenylseleno ligands in position 5' of deoxyribose ring of AZT molecule, named: 5'-(4-Chlorophenylseleno)zidovudine (derivative 1), 5'-(Phenylseleno)zidovudine (derivative 2) and 5'-(4-Methylphenylseleno)zidovudine (derivative 3) (Fig. 1).

Isolation of human erythrocytes: Venous human blood was collected into K_3EDTA vacuum tubes from healthy volunteers with age between 20 and 40 years. Erythrocytes were isolated by centrifugation of whole blood at 500 g for 10 min at room temperature. The erythrocytes portions were washed three times with buffered saline solution. The final suspension with the isolated erythrocytes was resuspended in appropriate buffer to give specific concentrations of hematocrit for each assay. Cells were immediately used for the tests.

Exposure of erythrocytes to AZT and Selenium-derivatives: Based in the peak plasma concentrations of AZT found in patients under therapy, samples of isolated erythrocytes were pre-incubated with AZT and

selenium derivatives at 10, 25, 50, 100, 200 and 500µM or vehicle (DMSO) for different incubation times at 37°C. After incubation, cells were used in *in vitro* assays.

Hemolysis measurement: For determination of cell hemolysis, washed erythrocytes (15% hematocrit) were treated with AZT and selenium derivatives in a phosphate buffer-saline medium for 3h, with slightly mixing every 30 min. Afterwards, samples were centrifuged at 750g for 10 min and resultant supernatant used to measure hemolisys (Godal & Heisto, 1981).

Osmotic fragility test: The ability of erythrocytes to resist hypotonic conditions was estimated according to the method described by (Godal & Heisto, 1981), by measuring hemolysis in varying concentrations of saline solution.

Reactive species generation by DHR staining: The fluorescence probe dihidrorhodamine 123 (DHR-123) was employed to detect the intracellular reactive species levels, following Royall and Ischiropoulos (1993).

Delta-aminolevulinate dehydratase (δ-ALA-D) activity: The activity of sulfhydryl enzyme δ-ALA-D was determined according the method proposed by Sassa (1982), by measuring the rate of porphobilinogen (PBG) formation with minor modifications. In order to investigate inhibition mechanisms, 500µM of Zinc (Zn^{2+}) and the reducing agent dithiothreitol (DTT) were simultaneously incubated with erythrocytes.

Non Protein Thiols (NPSH) Determination: The rate of NPSH oxidation was evaluated by measuring the disappearance of -SH groups based in a spectrophotometric method proposed by Ellman (1959) using the colored reagent 5′5′-dithio-bis(2-nitrobenzoic) acid (DTNB), which reacts with -SH groups.

Statistical analysis: All parameters were analyzed by One-way ANOVA followed by Bonferroni test when appropriate. $p<0.05$ was considered to indicate a statistically significant difference among groups.

Results/discussion: The derivative 1 to 500µM caused immediate hemolysis; osmotically erythrocyte membranes became more fragile after treatment with derivative 1 alone at concentrations of 100 and 200µM, and not with AZT and derivatives 2 and 3. In the treatment of erythrocytes with derivative 1 from 50µM, the percentage of DHR-positive cells increased significantly. Derivatives 1 and 2 caused a significant inhibition of δ-ALA-D activity, inhibition caused by derivative 2 was completely restored after addition of DTT, but this was not similar to derivative after treatment with Zn^{2+} or DTT. Therefore, derivative 2 does not have an affinity for thiol groups. Derivative 1 caused the reduction of the content of non-protein sulfhydryl groups (SH), in concentrations of 200 and 500µM.

Conclusion: Minor changes in the AZT structure can modify its capacity to induce

cytotoxicity as observed for derivatives 1 and 2 in erythrocytes. Only the selenium derivative 3 exhibited similar response patterns to that caused by the AZT nucleoside even at high concentrations. Therefore, the non-toxic forms of selenium compounds might minimize erythrocyte damages provoked by disorders in which oxidative stress/inflammation has been implicated in their ethiology. Within this perspective, derivative 3 appears to be a promising candidate.

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