Reduction of DNA-damaging effects of anti-HIV drug 3'-azido-3'-dideoxythymidine on human cells by ursolic acid and lignin biopolymer*

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In this study we verified our assumption that the genotoxicity of the effective anti-HIV drug 3'-azido-3'-dideoxythymidine (AZT) on human cells could be reduced by non-toxic concentrations of two antioxidants that occur frequently in nature (ursolic acid and lignin biopolymer). Cytotoxicity of these natural compounds, well-known by their antimutagenic effects, was evaluated by the trypan blue exclusion technique. Genotoxic activity of AZT was measured on the basis of AZT-induced single and double strand breaks to DNA in two histopathologically different types of human cells, hepatoma cells HepG2 and colonic cells Caco-2. Induction of DNA strand breaks was measured by the comet assay processed in parallel at pH≥13.0 (standard alkaline technique which enables to recognize single strand DNA breaks of different origin) and at pH=9.0 (neutral technique which enables to recognize double strand DNA breaks). As the level of AZT-induced double strand DNA breaks was rather low, protective effects of the antioxidants tested were evaluated only against AZT-induced single strand DNA breaks by the standard alkaline comet assay. Our findings showed that 1 h pre-incubation of cells with ursolic acid or lignin preceding to 3 h treatment of cells with AZT (3 mg/ml) significantly decreased in both cell types the level of AZT-induced single strand DNA breaks. Pre-incubation of HepG2 or Caco-2 cells with a mixture of both natural antioxidants did not increase the effects of individual treatments. This study confirms that AZT is genotoxic toward both used cell types of human origin and that ursolic acid and biopolymer lignin can protect the cells studied against genotoxic effect of AZT.

Key words: alkaline and neutral comet assay, human cells HepG2 and Caco-2, ursolic acid, lignin biopolymer

Zidovudine (3'-azido-3'-dideoxythymidine; AZT; ZDV; Retrovir®) represents the first nucleoside analogue approved for the treatment of HIV-1/AIDS. Unfortunately a successful reduction of the HIV virus incidence and transmission by this thymidine analogue could be connected with toxic side effect including carcinogenesis. It was found that AZT caused tumors in adult mice exposed to the drug or in several tissue types of mice fetuses exposed *in utero* [1–4]. The International Agency for Research on Cancer [5] classified therefore AZT as a group 2B carcinogen, designated as possibly carcinogenic to humans. Relatively few investigations have been carried out to characterize cytotoxic and DNA-damaging effects of this antiretroviral drug on human cells cultured *in vitro*. In the human lymphoblastoid cell line TK6, MENG et al [6] evaluated the potential mechanisms of AZT mutagenicity and carcinogenicity by quantifying the incorporation of AZT into cellular DNA by AZT radioimmunoassay. On the basis of a positive correlation between AZT-DNA incorporation and AZT-induced TK mutations the authors assumed that AZT incorporation into cellular DNA played a direct role in the genotoxicity and carcinogenicity of AZT. Cytotoxic effects of AZT on human placenta *in vitro* investigated COLLIER et al [7] who found that AZT induced apoptosis and altered levels of metabolizing enzymes in JEG-3 choriocarcinoma cells and primary explant cultures from human placentas. BIALKOWSKA et al [8] proved, that AZT

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causes in CD-1 Swiss mice oxidative damage in nuclear DNA of fetal liver tissue, which represents a target tissue for transplacental carcinogenesis. We described recently the nature and repair of DNA strand breaks induced by this antiretroviral drug in human hepatoma cells, human colonic cells and human embryonic lung fibroblasts [9].

Screening of substances that could prevent the anti-HIV drug-induced cellular and molecular damage is very important, as a major limitation in the use of AZT is the occurrence of severe side effects. Previously it was found that a synthetic compound glutapyrone, a glutamate containing 1,4-dihydropyridine (DHP) derivative, was capable to protect the self-aggregation of mitochondria accelerated by AZT [10]. The role of vitamins and minerals in reducing the symptoms of HIV infection was reviewed by ROMEYN [11]. We investigated in this paper the ability of ursolic acid (3-hydroxy-12urs-12en-28-oic acid) and lignin biopolymer to protect human DNA against damaging effects of AZT. Both compounds represent very frequent natural compounds. Ursolic acid belongs among compounds naturally found in various plants such as sea-weeds, the wax-like coatings of fruits and many medicinal herbs, e.g. rosemary, thyme, oregano and lavender. This pentacyclic triterpenoic acid which occurs in plants in the form of a free acid or an aglycone for triterpenoid saponins was long considered to be biologically inactive but in recent years, it has attracted the interest of medical scientists because of its pharmacological effects combined with a low toxicity [12]. The traditional uses of plants containing ursolic acid in folk medicine are multiple. The second natural compound tested, lignin, together with cellulose and hemicellulose, is a major constituent of the cell wall of plants and the most abundant organic polymer in the biosphere. Biosynthesis of lignin occurs by radical polymerization of p-cumaryl, coniferyl and sinapyl alcohols to form phenylpropane units, e.g. guaiacyl and syringyl, with hydroxyl and carbonyl substitutions [13].

Scientific objectives of this paper are relevant to prevention of potential adverse (genotoxic) effects of anti-HIV drug AZT as pre-clinical tests can not be performed on human beings and basic information on the effect of drugs could be acquired only from a broad spectrum of biological experiments using *in vitro*, *in vivo* or *ex vivo* approach.

Material and methods

Cell lines. Both human cell lines, Caco-2 and HepG2, were obtained from Prof. A.R. Collins (University of Oslo, Oslo, Norway). Cells were cultured at 37 °C in humidified atmosphere of 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM; Caco-2) or William's medium (HepG2) supplemented with 10% fetal calf serum and antibiotics (penicillin 200 U/ml, streptomycin 100 µg/ml and kanamycin 100 µg/ml).

3'-azido-3'-dideoxythymidine (zidovudin, AZT; ICN Biomedicals Inc., Ohio, USA) was dissolved immediately before use in culture medium (10 mg/ml) and further diluted with DMEM or William's medium to 2–5 mg/ml solutions.

Ursolic acid – extraction and isolation. The herb of sage (*Salvia officinalis* L., Lamiaceae) was collected at the Garden of Medicinal Plants, Faculty of Pharmacy, Comenius University in Bratislava. A voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University. Air-dried herb of *Salvia officinalis* L. (800 g) was powdered and extracted stepwise with petroleum ether, ether, chloroform, methanol, and diluted methanol (1:1). The filtered extracts were separately concentrated under diminished pressure at a water-bath temperature not exceeding 60 °C to dryness. The ethereal macerate (52.1 g) contained a mixture of triterpenoids showing a positive Liebermann-Burchard test was triturated with ether to give a green-white precipitate (31.4 g), which yielded β-ursolic acid (12.4 g) upon crystallization from ethanol. The mother liquors were chromatographed on a silica gel-packed column (deactivated with 10% of water) with chloroform and its mixtures with methanol. The content of fractions was monitored by TLC on Silufol® sheets (detection with sulfuric acid). The combined fractions 22 to 31 were crystallized from ethanol to afford further amounts of β-ursolic acid (0.45 g). The structure elucidation of β-ursolic acid and other compounds isolated from the herb of *Salvia officinalis* L. was reported by MAŠTEROVÁ et al [14]. Ursolic acid was dissolved immediately before use in dimethyl sulfoxide (DMSO; 1 M) and further diluted with DMEM or William's medium to $1x10^{-9}$–1 mM solutions for treatment of cells.

Lignin biopolymer, which represents a water-soluble sulfur-free lignin preparation of average molecular mass 2,000, was obtained by fractionation of hardwood hydrolysate (170 °C). It contained 19.1% OCH_3 and 0.05% ash. Gel permeation chromatography was performed on a column (53x8 cm) of Sephadex LH 60 using a mixture of dioxane and water containing 0.005 M of aqueous NaOH and 0.001 M of LiCl (7:3) as the eluant. Lignin was dissolved immediately before use in DMSO (10 mg/ml) and further diluted with DMEM or William's medium to 0.01–0.1 mg/ml solutions for treatment of cells.

Chemicals were obtained from the following sources: DMEM, William's medium, LMP (low melting point) agarose, trypan blue (0.4%) and fetal calf serum (FCS) from GIBCO BRL, Grand Island, NY; DMSO, trypsin, NMP (normal melting point) agarose, ethidium bromide and Triton X-100 from Sigma-Aldrich Co., St. Louis, MO. Other chemicals were of analytical grade from commercial suppliers.

Cytotoxicity assay (trypan blue exclusion). Caco-2 or HepG2 cells (exponentially growing in monolayer on glass vials) were exposed during 1 h to different concentrations of ursolic acid ($1x10^{-9}$–1 mM) or lignin (0.01–0.1 mg/ml) diluted in culture medium. Control cells were fed with fresh culture medium containing 0.5% DMSO. Then both control and treated cells were washed with complete medium and

cultured for 24 h in fresh culture medium, trypsinized, stained with trypan blue (0.4%) and the number of viable (uncolored) and dead (colored) cells was scored. The ratio of the number of viable/all cells gives the percentage of viable cells.

Single cell gel electrophoresis (SCGE = the comet assay). The procedure of SINGH et al [15] was followed with modifications made by SLAMEŇOVÁ et al [16], GÁBELOVÁ et al [17] and WOZNIAK and BLASIAK [18]. Briefly: a base layer of 1% NMP agarose (in PBS (Ca^{2+}- and Mg^{2+}-free)) was placed on pre-coated (1% NMP agarose in distilled H_2O) microscope slides. The tested cells suspended in 0.75% LMP agarose (in PBS (Ca^{2+}- and Mg^{2+}-free)) were spread on the base layer on each of three-five slides. A cover slip was added and the agarose was allowed to solidify. The cover slips were removed and the slides were placed in lysis mixture (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH=10.0 and 1% Triton X-100) at 4 °C for 1 h to remove cellular proteins. To distinguish between single and double strand DNA breaks unwinding and electrophoresis steps were performed in parallel using different conditions: slides were transferred to electrophoresis boxes containing solutions at pH≥13.0 (300 mM NaOH, 1 mM Na_2EDTA) or at pH=9.0 (100 mM Tris-HCl, 300 mM sodium acetate, pH adjusted to 9.0 by glacial acetic acid). They were kept in these solutions for unwinding time at 4 °C for 40 min (pH≥13.0) or 20 min (pH=9.0), resp. A current of 25 V (300 mA) was then applied for 30 min (pH≥13.0) or a current of 16.6 V (50 mA) for 60 min (pH=9.0), resp. The slides were removed, neutralized with Tris-HCl (0.4 M, pH=7.5) and stained with 20 µl ethidium bromide (EtBr, 5 µg/ml). Comets were analyzed under an Olympus fluorescence microscope. For each sample, 100 comets were scored by computerized image analysis (Komet 5.5, Kinetic Imaging Ltd., Liverpool, UK) for determination of DNA in the tail, which is linearly related to the frequency of single strand DNA breaks.

Data analysis. For the trypan blue exclusion technique mean ± standard deviation (SD) was calculated (three experiments with 2 replicate samples). The values of the single cell gel electrophoresis were expressed in this study as means ± SD from three experiments with 3–5 replicate samples. The significance of differences between samples was evaluated by Student's t-test – statistically different: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Results

Varying the pH during electrophoresis affects the type of strand breaks. When the lysed cells are subjected to electrophoresis at pH = 9.0 only double strand DNA breaks can be detected as this pH doesn't allow unwinding (dissociation) of single DNA strands. Under pH≥13.0 conditions the single strand DNA breaks and alkali-labile DNA lesions are detected. As it is evident from Figure 1, we found in both cell types a significant concentration-dependent increase of double strand DNA breaks, this increase was mildly more evident in HepG2 cells. Figure 2 shows the level of DNA strand breaks at the standard alkaline comet assay.

Viability of HepG2 and Caco-2 cells treated during 1 h with different concentrations of ursolic acid or lignin represent Figure 3 and Figure 4. For further studies we selected rather low concentrations of ursolic acid (0.001 mM) and lignin (0.05 mg/ml) which allowed approximately 80% survival of cells.

Figure 5 and Figure 6 represent the level of single strand DNA breaks in control cells, cells treated with ursolic acid (UA), lignin (L), AZT, and combinations of ursolic acid + AZT; lignin + AZT; and UA/L + AZT. In these experiments

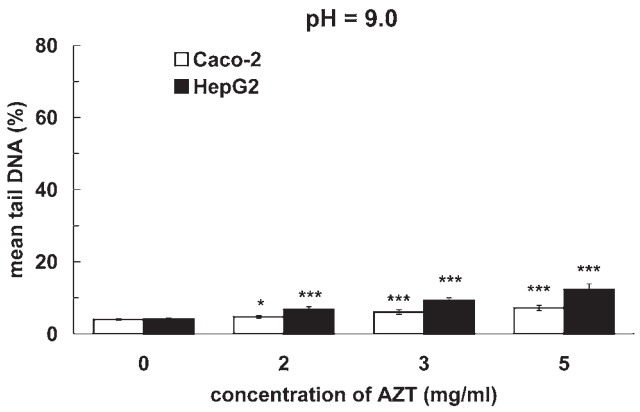

Figure 1. Level of double-strand DNA breaks in Caco-2 and HepG2 cells treated for 3 h with AZT (2–5 mg/ml). Individual samples of treated and control cells were processed by the comet assay at pH=9.0. Asterisks ($^{*}p<0.05$, $^{***}p<0.001$) show significant differences between control and treated samples. (Mean of three independent experiments ±SD).

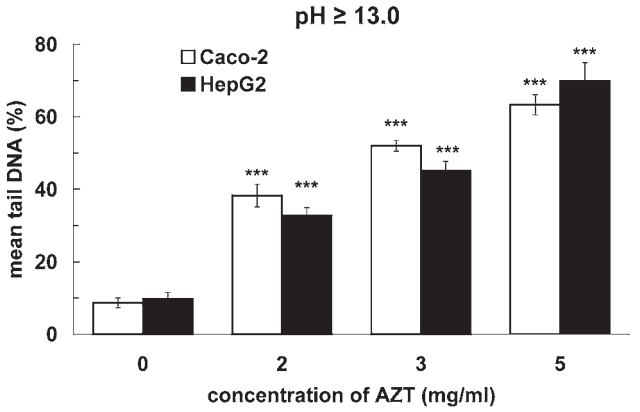

Figure 2. Level of DNA damage in Caco-2 and HepG2 cells treated for 3 h with AZT (2–5 mg/ml). Individual samples of treated and control cells were processed by the comet assay at pH≥13.0. Asterisks ($^{***}p<0.001$) show significant differences between control and treated samples. (Mean of three independent experiments ±SD).

we used the standard alkaline version of the comet assay (pH≥13.0) where the level of breaks (representing mixture of single strand DNA breaks and alkali-labile DNA lesions) was much higher than the level of double strand DNA breaks detected at pH=9.0. Protective effects of ursolic acid and lignin against DNA lesions induced by AZT in HepG2 cells are presented in Figure 5 and in Caco-2 cells in Figure 6. Both natural compounds reduced significantly the level of AZT-induced strand breaks in both cell types. Combination of ursolic acid and lignin did not show any synergistic (cumulative) protective effect.

Discussion

Evolution in HIV and AIDS therapy should not be focused only on novel therapeutic molecules targeting virus replication and integration but also on the proper combination of well-known and effective anti-HIV drugs with substances capable to prevent the anti-HIV drug-induced cellular and molecular damage. Protection against AZT-induced toxic effects by natural antioxidants has already been investigated by *in vitro* [11, 19] and *in vivo* [20–22] approaches. We decided to study ability of two well known natural antioxidants and antimutagens (ursolic acid and lignin biopolymer) to protect human DNA against damaging effects of AZT in *in vitro* conditions. To the best of our knowledge similar study has never been done.

In our previous paper [9] we evaluated in AZT-treated human cells of different origin induction of DNA strand breaks and their repair using the comet assay with pH variations (pH≥13.0 and pH=12.1). We found that AZT induced in all cell types studied repairable single strand DNA breaks, but in comparison with colonic cells the kinetics of DNA repair was more proficient in hepatoma and lung cells. TOMICIC et al [23] found that numerous nucleosides are highly potent not only in the induction of DNA single strand breaks but also of DNA double strand breaks. We included therefore into this study also measurement of double strand DNA breaks. As it is evident from Figure 1, we found in both cell types a significant concentration-dependent increase of double strand breaks which could arise from AZT-induced DNA degradation connected with the cell death. Figure 2 shows the level of DNA strand breaks at a standard alkaline comet assay. With regard to the yield of DNA strand breaks, which was more evident under the alkaline conditions, the protective effects of ursolic acid and lignin were tested by the standard alkaline comet assay. An appropriate and harmless treatment regimen of cells with ursolic acid and lignin was established on the basis of the viability of cells treated during different time intervals with different concentrations of ursolic acid or lignin (data not shown). For studying of protective effects of both natural compounds against DNA-damaging effects of AZT we selected 1 h incubation of cells with 0.001 mM of ursolic acid or 0.05 mg/ml of lignin which enabled approximately 80% of survival (Fig. 3, Fig. 4).

Many of therapeutic effects of ursolic acid were described [24]. Ursolic acid has been shown to inhibit various stages of tumor development [25, 26] and to induce tumor cells differentiation [27]. It effectively inhibits angiogenesis [28], invasion of tumor cells and metastasis [26]. Besides these effects, it has been reported to have anti-inflammatory [29], hepatoprotective [30], gastroprotective [31], cardiovascular [32], hypolipidemic [31], antiatherosclerotic [32], and immunoregulatory effects [33]. Very important property of ursolic acid is its potent anti-HIV activity operative through a confirmed mechanism of inhibiting dimerization of HIV-1 protease [34, 35]. The complex beneficial effects of ursolic acid were described recently [36].

Lignin has an anti-immunodeficiency virus activity and anti-influenza virus activity [37, 38] and is an effective binder of bioacid products of cholesterol degradation [39] and of nitrosamines [40]. The adsorptive ability of lignin is

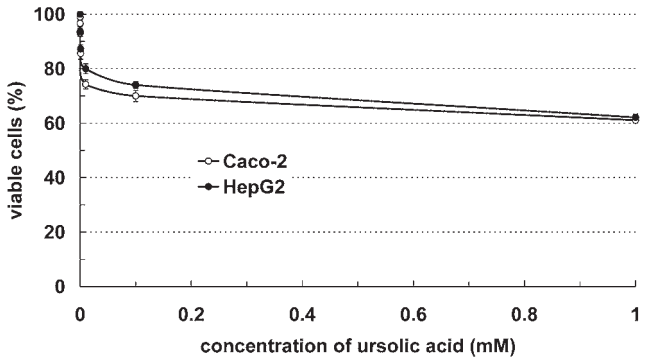

Figure 3. Effect of ursolic acid on viability of Caco-2 and HepG2 cells. Caco-2 (○) and HepG2 (●) cells were treated with different concentrations of ursolic acid ($1x10^{-9}$–1 mM) or with 0.5% DMSO for 1 h and after the treatment viability was measured by trypan blue exclusion. (Mean of three independent experiments ±SD).

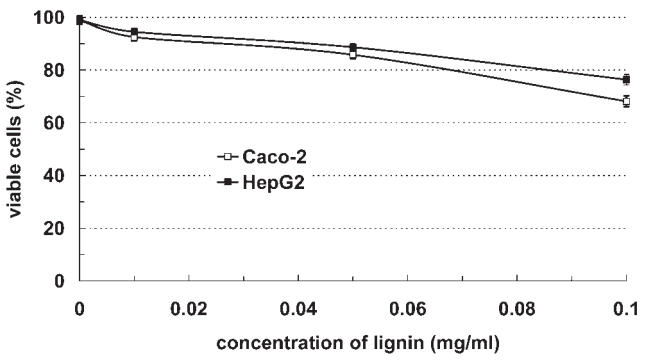

Figure 4. Effect of lignin on viability of Caco-2 and HepG2 cells. Caco-2 (□) and HepG2 (■) cells were treated with different concentrations of lignin (0.01–0.1 mg/ml) or with 0.5% DMSO for 1 h and after the treatment viability was measured by trypan blue exclusion. (Mean of three independent experiments ±SD).

supplemented with its antioxidant activity owing to the presence of unique hindered phenolic hydroxyl groups, which act as a stabilizer of reactions induced by oxygen and its radical reduction products [41, 42]. We confirmed this dual effect of lignin in our previous *in vitro* experiments. Lignin reduced genotoxic effects of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as well as H_2O_2 in stabilized cell lines VH10, Caco-2 and V79 cultured *in vitro* [43], genotoxic effects of visible light-excited methylene blue in V79 cells [44], the level of 6-TG^r mutations in MNNG-treated V79 cells [45], the frequency of mutations and SOS response induced by 4-nitroquinoline-N-oxide in *Escherichia coli* PQ37 cells [46] as well as oxidative DNA lesions in testicular cells and lymphocytes of Sprague-Dowley rats *in vitro* and *ex vivo* [47]. Numerous studies showed that lignin and its metabolites have anticarcinogenic properties [42, 48, 49].

With help of DNA repair enzymes Endo III and Fpg we ascertained in our previous paper [9] that 20–25% of single strand DNA breaks induced in human cells by AZT represented the repair enzyme sensitive sites, i.e. the oxidative DNA lesions. Approximately the same amount of strand breaks was eliminated by pre-treatment of HepG2 and Caco-2 cells with lignin (Fig. 5, Fig. 6). This result suggests that lignin eliminates mainly oxidative DNA lesions. Ursolic acid was more effective, as it reduced approximately 50% of AZT-induced strand breaks. This can be explained by the fact that ursolic acid belongs among the phytochemicals which in addition to antioxidative effects acts also as detoxification agents for carcinogens by enhancing the activity of detoxification enzymes [50, 51].

An important point when assessing the antioxidant activity of plant antioxidants is to consider their interaction with other antioxidants. Combination of two or more antioxidants could have a synergistic effect and provide superior protection to that derived from each agent alone. Figure 5 and Figure 6 show the effect of pre-incubation of cells with both natural components tested. It is evident that the cells pre-incubated with combination of ursolic acid and lignin did not manifest any synergistic protective effect against AZT. Their resistance against AZT was similar to the resistance of cells pre-incubated with lignin alone. It seems that the endogenous presence of lignin biopolymer in the cells tested makes impossible manifestation of protective effect of ursolic acid. An absence of syner-

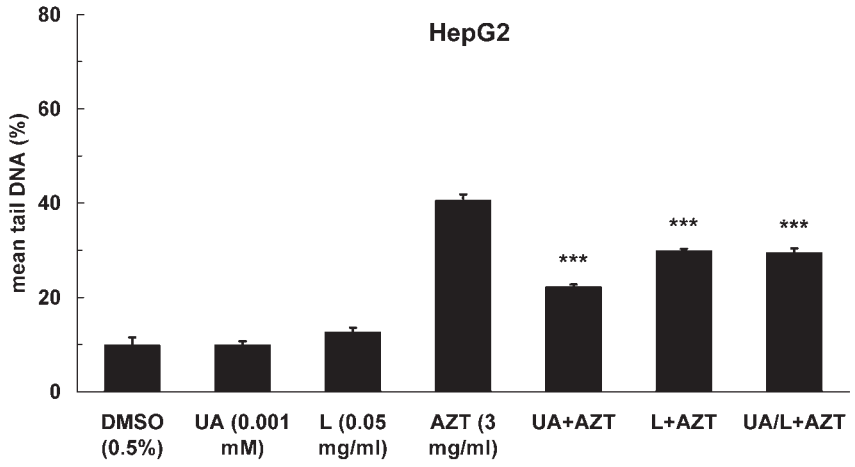

Figure 5. Effect of ursolic acid (UA), lignin (L) or ursolic acid/lignin (UA/L) pre-treatment on AZT-induced DNA damage in HepG2 cells. HepG2 cells were treated with 0.5% DMSO, ursolic acid (0.001 mM), lignin (0.05 mg/ml) or ursolic acid/lignin (UA/L) for 1 h, washed with William's medium, and exposed for 3 h to 3 mg/ml AZT. Individual samples of control (0.5% DMSO) and treated cells were processed by the comet assay at pH≥13.0. ***p<0.001 refers to differences between cells treated with 3 mg/ml AZT alone and cells pre-treated with UA, L or UA/L and then exposed to AZT. (Mean of three independent experiments ±SD).

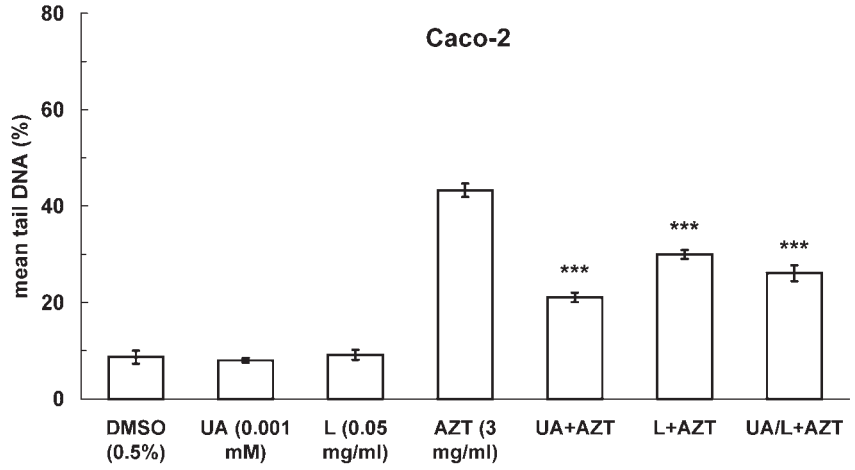

Figure 6. Effect of ursolic acid (UA), lignin (L) or ursolic acid/lignin (UA/L) pre-treatment on AZT-induced DNA damage in Caco-2 cells. Caco-2 cells were treated with 0.5% DMSO, ursolic acid (0.001 mM), lignin (0.05 mg/ml) or ursolic acid/lignin (UA/L) for 1 h, washed with DMEM, and exposed for 3 h to 3 mg/ml AZT. Individual samples of control (0.5% DMSO) and treated cells were processed by the comet assay at pH≤13.0. ***p<0.001 refers to differences between cells treated with 3 mg/ml AZT alone and cells pre-treated with UA, L or UA/L and then exposed to AZT. (Mean of three independent experiments ±SD).

gistic effect was found also in combinations of other antioxidants, e.g. ascorbic acid and isoflavones [52].

Overall, the results presented here demonstrate that AZT is genotoxic toward both used cell types of human origin and that ursolic acid and lignin biopolymer can protect the cells studied against DNA-damaging effects of AZT.

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