Effects of Cytokinins, Cytokinin Ribosides and their Analogs on the Viability of Normal and Neoplastic Human Cells

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Abstract. *We examined the effects of some cytokinins and cytokinin ribosides including a series of adenosine analogs differently substituted in the N^6 position, along with some hypoxanthine derivatives on the viability of normal and neoplastic human cells. Cytokinins such as trans-zeatin, isopentenyladenine and benzyladenine do not show any effect, while cytokinin ribosides such as trans-zeatin riboside, isopentenyladenosine, and benzylaminopurine riboside impair the viability of normal and neoplastic cells, apart from colon carcinoma LoVo cells.*

Cytokinins are important purine derivatives that serve as hormones that control many processes in plants (1). Chemically, known natural cytokinins are N^6-substituted adenines and their riboside, ribotide, and glycoside conjugates. The diversity of the N^6 substituents is the origin of the different cytokinin types. Cytokinins were discovered as factors that promote cell division in tobacco tissue cultures (2) and have been shown to regulate several other developmental events, such as de novo bud formation, release of buds from apical dominance, leaf expansion, delay of senescence, promotion of seed germination, and chloroplast formation (3). Naturally occurring cytokinins are mainly adenine derivatives, such as isopentenyladenine (Figure 1, 1b) and *trans*-zeatin (Figure 1, 5b), whereas synthetic cytokinins include several adenine analogs, such as kinetin and 6-benzyladenine (Figure 1, 3b) (4). Although cytokinins are known to have pronounced effects on plant development, little is known about their precise mechanisms of action. Cytokinins induce callus to redifferentiate into adventitious buds (5). Callus is clusters of dedifferentiated plant cells that are immortal and proliferate indefinitely in a disorganized manner, just like human cancer cells (6). Because there are some similarities between the phenotype of cancer and callus cells, cytokinins may also affect the proliferation and differentiation of human cancer cells, possibly through common signal transduction systems.

The only known cytokinin existing in animal cells is isopentenyladenosine (iPA; Figure 1, **1a**). It has been detected in the cytosol of many eukaryotic and prokaryotic cells as a free compound or bound to tRNA (7).

Modified adenine or adenosine derivatives are mainly found in tRNA and originate from cellular RNA breakdown. These derivatives were detected in urine and shown to be significantly elevated in the urine of patients with lung carcinoma and non-Hodgkin's lymphoma in comparison to healthy individuals (8, 9).

We have previously demonstrated that the antiproliferative activity of these molecules in human bladder carcinoma cells is optimal in N^6-substituted adenosine analogs with side chains containing at least three carbon atoms. We have also shown that modifications of the sugar moiety generate inactive molecules (10-12).

To continue our studies on the relation between structure and function of iPA (10-12), we focused our attention on natural and synthesized cytokinins and cytokinin ribosides, including a series of adenosine analogs differently substituted in the N^6 position, along with some hypoxanthine derivatives (Figure 1). These molecules were tested on normal diploid cells and on different tumor cell lines, which represent valuable preclinical models to identify potential therapy targets and pharmacologically useful compounds. In particular, we utilized one of the most used viability/ cytotoxicity tests, namely the neutral red uptake assay, which provides a quantitative estimation of the viable cells in a culture (13).

Materials and Methods

Reagents. N^6-Isopentenyladenosine (**1a**), N^6-isopentenyladenine (**1b**), *trans*-zeatin riboside (**5a**), *trans*-zeatin (**5b**), 6-chloropurine riboside, 6-chloropurine, all available starting amines, the other reagents and all solvents were purchased from Sigma-Aldrich (St.

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Louis, MO, USA). Organic solvents were dried in the presence of appropriate drying agents and were stored over suitable molecular sieves.

O^6-Isopentenylinosine (**2a**), *N*^{6}-benzyladenosine (**3a**), *N*^{6}-isopentyladenosine (**6**), *N*^{6}-butyladenosine (**7**), *N*^{6}-allyladenosine (**8**) were prepared and purified as described previously (11, 12). Purity of all compounds (≥99%) was verified by thin layer chromatography, nuclear magnetic resonance and mass spectrometry measurements. Structures of the aforementioned chemicals are presented in Figure 1.

All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Well plates were purchased from Euroclone (Pero, Milano, Italy).

Synthesis of O^6*-isopentenylhypoxanthine (**2b**).* To a suspension of sodium hydride (100 mg of a 55-65% oil dispersion, 2.3 mmol) in dry tetrahydrofuran (4 ml) cooled with an ice bath, 3-methyl-2-buten-1-ol (90 mg, 1.0 mmol) was added slowly. After 20 min, 6-chloropurine (100 mg, 0.65 mmol) was added and the solution was refluxed for 24 h. After removal of the solvent under vacuum, the residue was dissolved in a 10% solution of NH_4Cl and extracted with dichloromethane and ethyl acetate. Purification by column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) afforded 100 mg of product as a white solid (0.49 mmol, 75%, R_f=0.54 in CH_2Cl_2/MeOH 9:1). The physicochemical properties were similar to those previously described (14).

Synthesis of N^6*-benzyladenine (**3b**).* To a solution of 6-chloropurine (100 mg, 0.65 mmol) in absolute EtOH (5 ml), Et_3N, (200 mg, 2.0 mmol) and benzylamine (215 mg, 2.0 mmol) were added. The mixture was stirred at 80˚C for 3 h, cooled to room temperature and the solvent was removed under vacuum. The crude residue was crystallised from MeOH. White solid, 140 mg (0.62 mmol, 95%, R_f=0.49 in CH_2Cl_2/MeOH 9:1). The physicochemical properties were similar to those previously described (15).

Synthesis of O^6*-benzylinosine (**4a**).* To a solution of inosine (200 mg, 0.74 mmol) in dimethylformamide (4 ml) were added K_2CO_3 (300 mg, 2.3 mmol) and benzylbromide (720 mg, 4.2 mmol). After stirring at 150˚C for 2 h, the mixture was filtered on a celite pad and the solvent was evaporated under vacuum. Column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) afforded 180 mg (0.5 mmol, 68%, R_f=0.28 in CH_2Cl_2/MeOH 9:1) of desired product as a white solid; the physicochemical properties were similar to those previously described (16).

Synthesis of O^6*-benzylhypoxanthine (**4b**).* This compound was obtained as described for **2b**, starting from benzyl alcohol (110 mg, 1.0 mmol), 6-chloropurine (100 mg, 0.65 mmol) and sodium hydride (100 mg of a 55-65% oil dispersion, 2.3 mmol). Column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) afforded 100 mg of desired product (0.44 mmol, 68%, R_f=0.59 in CH_2Cl_2/MeOH 9:1) as a white solid. The physicochemical properties were similar to those previously described (17).

Cell culture. Primary human umbilical vein endothelial cells (HUVECs) were cultured in M199 containing 10% fetal bovine serum, 1 mM glutamine, 1 mM penicillin and streptomycin, endothelial cell growth factor (ECGF; 150 μg/ml), 1 mM sodium pyruvate and heparin (5 units/ml). LoVo cells grew in HF12, the other cell lines in DMEM, all supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine and 1 mM penicillin and streptomycin, in 5% CO2 at 37˚C. All the reagents for cell culture were from Gibco-Invitrogen (San Giuliano Milanese, Italy). Stock solutions of iPA and the different compounds were prepared in dimethylsulfoxide (DMSO) and kept at –20˚C. Appropriate dilutions of the compounds were freshly prepared in culture medium just prior to the assays. The controls used media with final concentrations of DMSO of 0.01%.

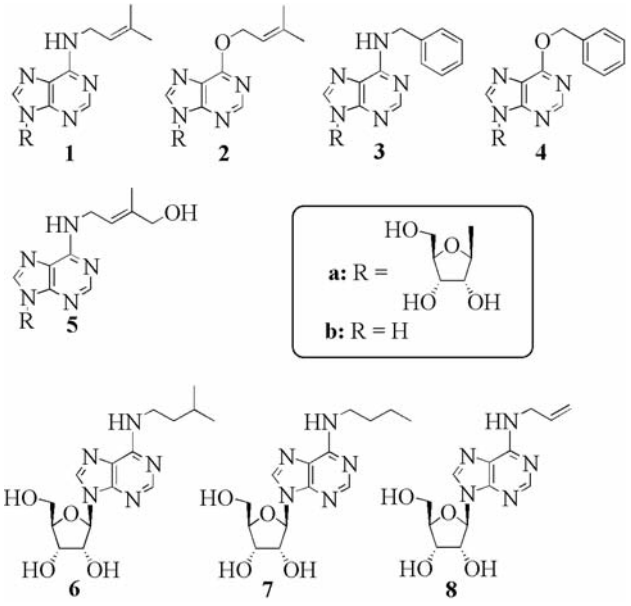

Figure 1. *Chemical structures of examined cytokinins, cytokinin ribosides and analogs: N*^{6}*-isopentenyladenosine (**1a**), N*^{6}*-isopentenyladenine (**1b**), O*^{6}*-isopentenylinosine (**2a**), O*^{6}*-isopentenylhypoxanthine (**2b**), N*^{6}*-benzyladenosine (**3a**), N*^{6}*-benzyladenine (**3b**), O*^{6}*-benzylinosine (**2a**), O*^{6}*-benzylhypoxanthine (**2b**), trans-zeatin riboside (**5a**), trans-zeatin (**5b**), N*^{6}*-isopentyladenosine (**6**), N6-butyladenosine (**7**), N*^{6}*-allyladenosine (**8**).*

Cell viability/cytotoxicity. Neutral red uptake assay was used to estimate cell viability/cytotoxicity in response to iPA and its derivatives (13). Briefly, 16 h after seeding, iPA and its derivatives at 10 μM were added. After 3 days, neutral red was added to the medium to a final concentration 50 μg/ml. After 2 h, the wells were washed with PBS and fixed by adding destaining solution containing 50% ethanol and 1% acetic acid in water. The optical density was read at 540 nm in a microtiter plate reader spectrophotometer using blanks containing no cells as a reference. Results are shown as a percentage that of controls.

Clonogenic assay. T24 cells (5000 per well) were seeded into 6-well plates and cultured in the presence of iPA or its derivatives at 10 μM. The media were replaced every 2 or 3 days and supplemented with fresh compounds. After culture for a week, colonies were fixed in 0.5% crystal violet in methanol and extensively washed. The plates were then photographed. Plates were stained with crystal violet and cells colonies (>50 cells) were counted and scored by Image J Quantification Software.

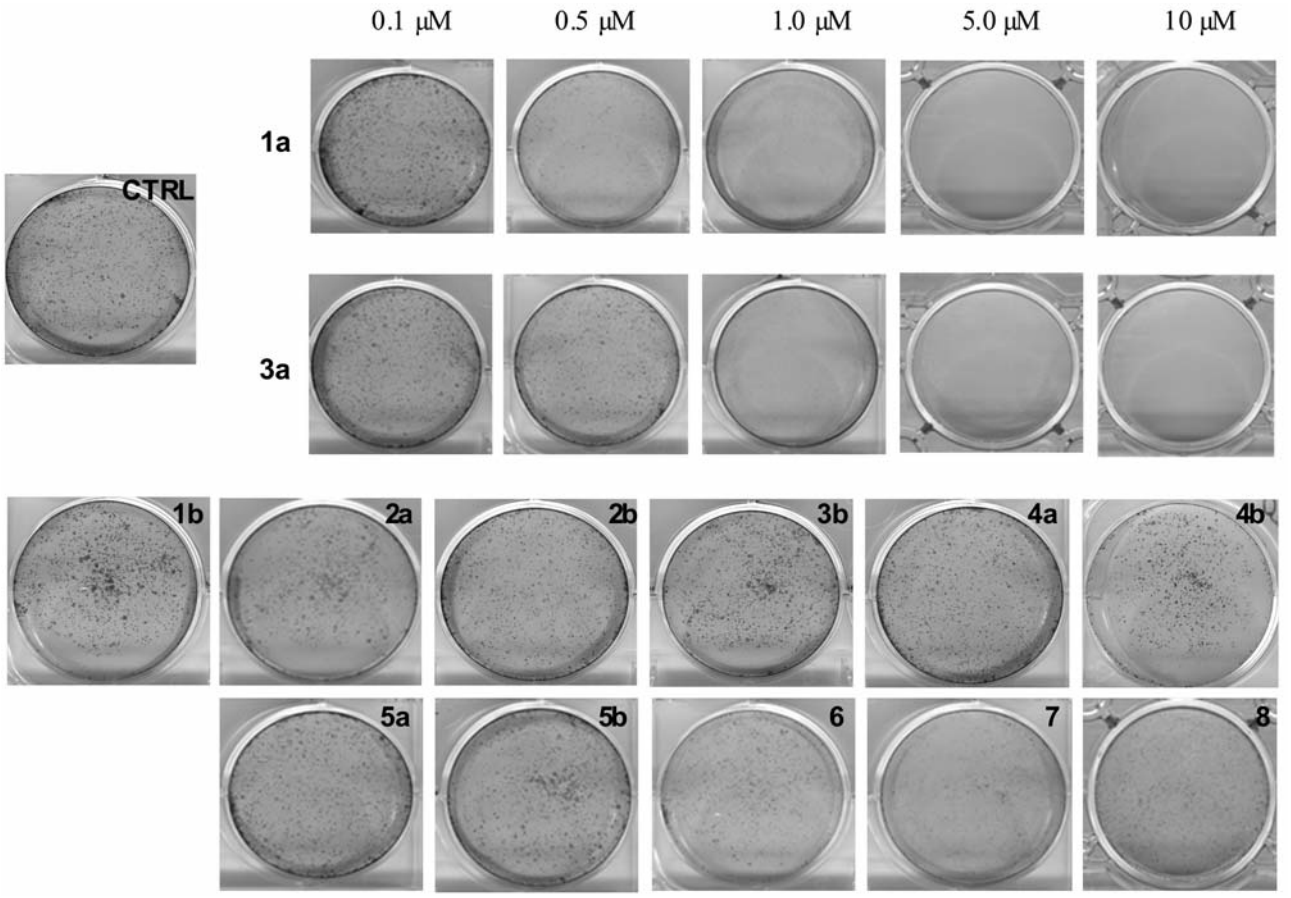

Figure 2. *Clonogenic assay on T24 cells treated with iPA and derivatives. T24 cells were seeded at very low density and treated with compound **1a** and **3a** at concentrations between 0.1 and 10 μM. The remaining compounds were used at 10 μM. CTRL is the control. After a week, the cells were stained and photographed.*

Statistical analysis. All the experiments were performed at least 3 times in triplicates and data are shown as the mean±standard deviation. Statistical significance was determined using Student's *t*-test. Values of $p<0.05$ were considered significant.

Results

In addition to their essential roles in the growth and development of plants, cytokinins have various effects in man and animals at both cellular and whole organism levels (18-20). Hence, cytokinins and their derivatives have many potential therapeutic applications, including possible efficacy in the treatment of proliferative diseases such as cancer. The anticancerous activity of cytokinins in a variety of cultured cell lines has been documented (21, 22). Here, we report studies on the cytotoxic activity of some cytokinins, citokinin ribosides and their analogs on a panel of different neoplastic cell lines and on primary endothelial cells. The tested compounds include some known natural cytokinins and cytokinin ribosides, such as compounds **1a** and **b**, **3a** and **b**, 5a and b, and structural variants in which the nitrogen atom at the purine 6 position was substituted with an oxygen atom, such as compounds **2a** and **b**, **4a** and **b**. We also tested compounds **6**, **7** and **8** which, as reported in our previous studies (12) on T24 cells, had shown a certain anti-proliferative activity.

We first evaluated the different compounds by clonogenic assays on bladder carcinoma T24 cells. Figure 2 shows that compounds **1a** and **3a** inhibited clonogenity with similar dose dependence. We confirm that compounds **6**, **7** and **8** retained an inhibitory activity when used at 10 μM, as previously described (12), while all the other compounds used at this concentration had no effect. We then evaluated the response of various cell types to the different compounds by neutral red assay. Only colon carcinoma LoVo cells were insensitive to all the molecules, including iPA (Figure 3A). On the contrary, compounds **1a** and **3a** significantly impaired the viability of HUVECs, bladder carcinoma T24 and

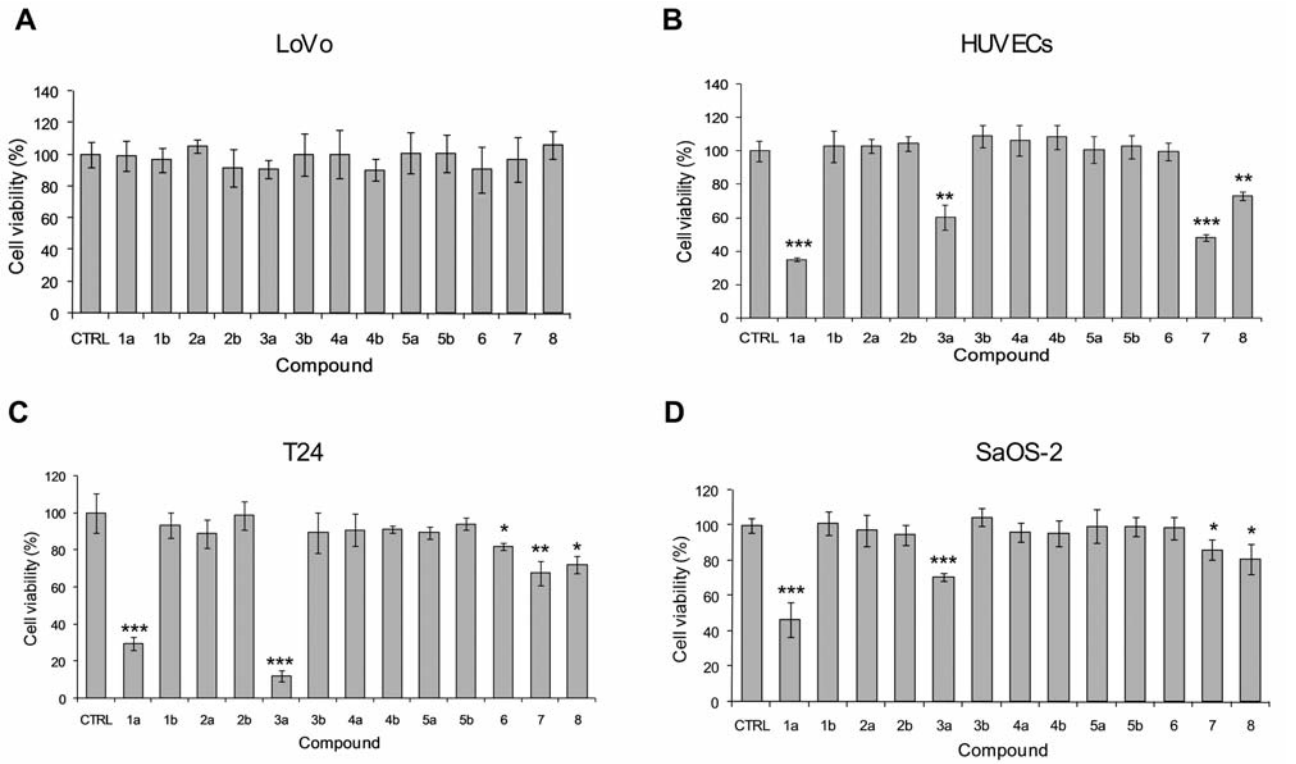

Figure 3. *Neutral red uptake assay on normal and neoplastic cells treated with different compounds. Viability was evaluated on LoVo (A), HUVECs (B), T24 (C) and SaOS-2 (D) cells exposed to 10 μM of iPA or derivatives for 72 h. The experiments were performed in triplicate at least three times. Data are shown as the mean±standard deviation. Significantly different from the control (CTRL) at *p<0.05; **p<0.01; *** p<0.001.*

osteosarcoma SaOS-2 cells (Figure 3 B-D). In addition, molecules **7** and **8** and, in the case of T24, **6**, significantly impacted on cell viability (Figure 3 B-D). We also investigated the effect of these molecules on ovarian carcinoma sensitive A2780 or resistant A2780 CP to cisplatin (25). We showed that cisplatin-resistant cells were more sensitive to the cytotoxic effect of **1a**, **3a**, **6** and **7** than cisplatin-sensitive cells (Figure 4).

Discussion

We prepared a series of cytokinin ribosides (**1a**, **3a**, **5a**) and cytokinins (**1b**, **3b**, **5b**), including a series of adenosine analogs differently substituted in the N^6 position (**6**, **7**, **8**), along with some inosine and hypoxanthine derivatives (**2a**, **4a**, **2b**, **4b**) by developing efficient synthetic routes. Only a few tested compounds showed cytotoxicity on most of the cells studied, including normal endothelial cells. However, we have identified a cell line, colon carcinoma LoVo, which is insensitive to iPA and its derivatives. To our knowledge, this is the first cell line which does not respond to iPA. Further studies at the molecular level are necessary to understand the mechanisms of LoVo cell resistance to iPA. We also show that ovarian carcinoma cells which are resistant to cisplatin, a drug currently used to treat this neoplasia, are significantly more sensitive to iPA and some derivatives than are cisplatin-sensitive cells. Although more studies are required, we hypothesize that our finding might open new perspectives in the approach to cisplatin-resistant ovarian cancer. It is noteworthy that osteosarcoma SaOS-2 cells are also sensitive to **1a** and **3a**. Osteosarcoma is the most common type of primary bone cancer in children and adolescents and often relapses, resulting in an unsatisfactory outcome (26). It is therefore important to individualize novel potential therapeutic options to control the disease and prolong survival; for this purpose, cytokinins might be of interest. Similar conclusions can be drawn for bladder carcinoma T24 cells, since these cells have provided important insights into bladder tumor progression events and metastatic dissemination.

Our data show that the absence of a sugar moiety or substitution of the nitrogen atom at the purine 6 position with an oxygen atom drastically inhibited iPA activity *in vitro*. Because benzyl derivative **3a** had a similar inhibitory activity to that of iPA (**1a**), it would seem that π-π electron interaction is fundamental for the cytotoxicity exhibited by

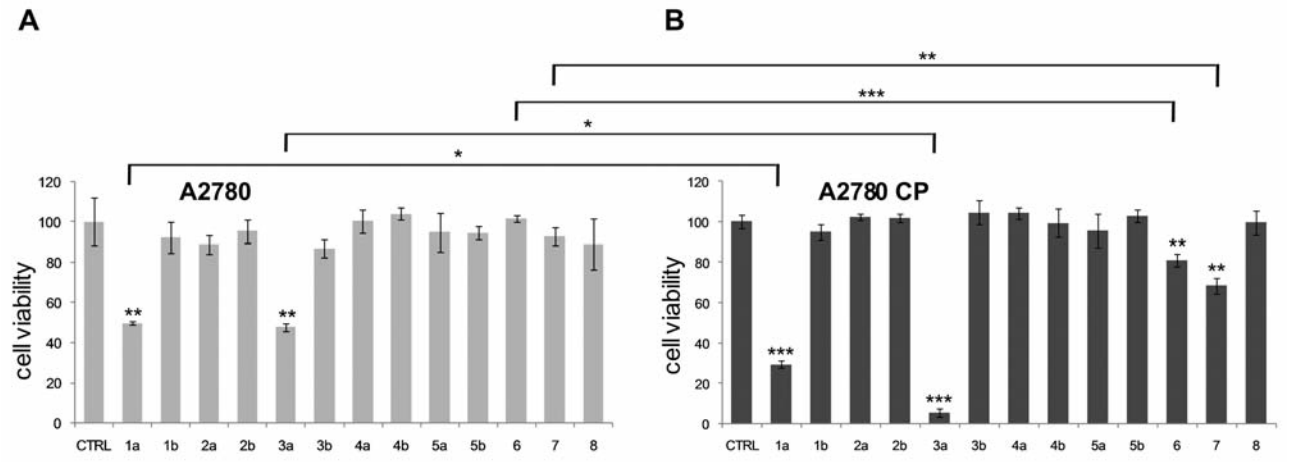

Figure 4. *Neutral red uptake assay on cisplatin-sensitive A2780 cells and cisplatin-resistant A2780 CP cells. Viability was evaluated on cells exposed to 10 μM of iPA or derivatives for 72 h. Statistical analysis was performed as described in the Materials and Methods. Significantly different from the control (CTRL) (or respective sensitive cells) at *(#)p<0.05; **(##)p<0.01; ***(###)p<0.001.*

these compounds. In addition, compound **3a** was endowed with significant cytotoxic activity in all cells, apart from LoVo cells, and can be viewed as a novel lead compound for further modifications.

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