Original Article

In Vitro Evaluation of the Cytotoxicity and Genotoxicity of Resorcylidene Aminoguanidine in Human Diploid Cells B-HNF-1

(cytotoxicity test / micronucleus test / resorcylidene aminoguanidine)

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Abstract. RAG belongs to appropriate inhibitors of protein glycation, i.e. formation of advanced glycation end products, which are thought to be responsible for some complications of DM, including neuropathy, angiopathy, retinopathy and nephropathy. In the present study authors have evaluated the genotoxic effect of RAG on the cell culture of human neonatal fibroblasts (B-HNF-1) in regard to its potential clinical application as inhibitor of advanced glycation end products in relationships to the pathogenesis of chronic diabetic complications. The direct contact cytotoxicity assay and micronucleus test were performed. The results showed that RAG in the concentration range of 1×10^{-4} to 1×10^{-6} mol.l^{-1} did not induce any changes in the morphology of exposed B-HNF-1 cells. The frequency of micronuclei was not significantly increased as well. The inhibitive effect of resorcylidene aminoguanidine was directly proportional to its concentration. It can be concluded that RAG at the selected concentrations has an inhibitive effect on proliferation of the treated cells and, at the same time, does not display any genotoxic effects on B-HNF-1 cells.

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Abbreviations: AG – aminoguanidine, AGE – advanced glycation end product, DM – diabetes mellitus, IC – intact control, MMC – mitomycin C, MNT – micronucleus test, PAG – pyridoxalphosphate-aminoguanidine adduct, PBSa – phosphate-buffered saline without calcium and magnesium, PC – positive control, RAG – resorcylidene aminoguanidine, SC – solvent control.

Introduction

It is known well that formation of advanced glycation end products (AGEs) of proteins plays a crucial role in the pathogenesis of diabetic complications including neuropathy, angiopathy, retinopathy, nephropathy and cataract. The first step of glycation is a nucleophilic additive reaction between free amino groups of proteins and the aldehyde group of glucose. The intermediate products (Schiff bases) are stabilized by an Amadori rearrangement, which is further followed by a spontaneous reaction leading to the formation of stable AGEs (Brownlee, 2001). Much effort has been devoted to finding an appropriate glycation inhibitor for diabetic complication treatment (Figarola et al., 2003; Metz et al., 2003; Huang et al., 2005).

One of the most studied inhibitors of protein glycation is aminoguanidine (AG), which acts by a combination of its antioxidant capability and selective scavenging of carbonyl reactive intermediates of both, the early and advanced glycation (Brownlee et al., 1986). It was shown that AG reacts with pyridoxalphosphate (vitamin B_6), causing production of pyridoxalphosphate-aminoguanidine adduct (PAG). This results in deficiency of this vitamin *in vivo* (Taguchi et al., 1998). Several studies have been performed to evaluate the properties of PAG and its capability to act as an inhibitor of protein glycation. It has been suggested that PAG does not influence the level of vitamin B_6 in the liver and kidneys and that it is a more acceptable inhibitor of protein glycation and formation of AGEs than AG (Taguchi et al., 1999; Miyoshi et al., 2002). Another promising inhibitor of AGE formation is resorcylidene aminoguanidine – RAG (Fig. 1) (Jakuš et al., 1999; Liptáková et al., 2002). RAG is an adduct of aminoguanidine and resorcinaldehyde and it has properties similar to PAG, including preserving pyridoxalphosphate levels. It was shown that RAG normalizes fluidity and potential in the membranes of erythrocytes in diabetic patients (Waczulíková et al.,

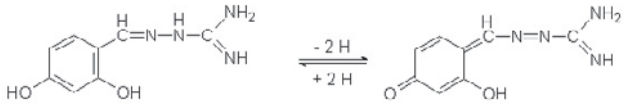

Fig. 1. Redox (antioxidant) system of RAG (pK = 7.34)

2000a, b). In addition, RAG possesses antimutagenic and bacteriostatic properties (Onuska et al., 1996).

Whenever a new chemical compound is introduced into pharmacology, its safety must be first demonstrated. Evaluation of the potential adverse health and environmental effects is accomplished largely by tests involving *in vitro* cytotoxicity and genotoxicity assays. To detect a cytotoxic effect, the direct contact method has been proposed because of its high sensitivity. Genotoxic properties of various chemical compounds can be investigated by the micronucleus test (MNT) in mammalian or rodent cell lines (Director et al., 1996; Meintieres et al., 2003). MNT is accepted as a short-term test for quick detection of substances that can disturb the cell division process. It was shown that there is a linear correlation between the level of chromosomal damage and formation of micronuclei (Beetstra et al, 2005). In the case of a positive MNT and significant increase of numerical and structural chromosomal aberrations, it is recommended to perform a fluorescent plus Giemsa staining technique in order to identify DNA damage in the exposed cells (Goto et al., 1975).

To our knowledge, no studies focused on the evaluation of cytotoxicity of RAG *in vitro* have been performed. This study presents the results obtained from the *in vitro* cytotoxicity testing and from the micronucleus test in cell culture of human neonatal fibroblasts (B-HNF-1) influenced by RAG with regard to its potential use in the pharmacologic treatment of diabetic complications.

Material and Methods

Resorcylidene aminoguanidine synthesis

RAG was synthesized by a condensation reaction according to the previously published method (Čársky et al., 1978). Stock solution of RAG was prepared by dilution in phosphate-buffered saline without calcium and magnesium (PBSa) with pH = 7.2. The prepared solution had a concentration of 1×10^{-1} mol.l^{-1} and was filtered with a single-use 0.22 μm microfilter (Millipore, Billerica, MA) before use.

Cell culture

Human neonatal diploid cell line (B-HNF-1) was established on September 14, 2004, from part of a skin excision submitted for histological examination from a 2-month-old Caucasian male patient using the tissue fragment technique. The cells were isolated in the Laboratory of Tissue Engineering at the Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University, Bratislava.

The modal chromosome number of the cell line is 46, population doubling time is 24 hours, and the cells have a normal diploid male karyotype. The adhering cells have fibroblastoid morphology. In the third passage, part of the cell suspension was tested for the presence of mycoplasma using polymerase chain reaction – PCR (Ossewaarde et al., 1996). On the following day, after a negative mycoplasma test, cells were trypsinized, counted in a haemocytometer, and tested for viability using a 0.5% (w/v) solution of trypan blue in water (Lachema, Brno, Czech Republic) diluted 1 : 1 with the cell suspension (Strober et al., 1991). The test result yielded 97% cell viability.

Cytotoxicity test

B-HNF-1 cells for testing cytotoxicity were thawed and evaluated in culture for viability using the trypan blue exclusion test. Then they were plated into 35 mm plastic Petri dishes (Falcon, San Jose, CA) in medium E-MEM, supplemented with 10% foetal bovine serum (FBS) and gentamycin at a final concentration of 100 μg.ml^{-1}. The cell culture was maintained in a humidified atmosphere containing 5% CO_2 in air at 37 °C. The culture medium was refreshed twice a week.

When the cells reached confluence, they were enzymatically detached by 0.5% trypsin/EDTA solution. The cell suspension was seeded into Petri dishes at the density of 1.25×10^5 .ml^{-1}cells. Subsequently, the cells were exposed to increasing concentrations of RAG (1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}, 1×10^{-3} and 1×10^{-2} mol.l^{-1}). E-MEM was used as an intact control (IC) and PBSa as a solvent control (SC). Cells were incubated in a CO_2 incubator for five days. Morphology, density and behaviour of the exposed cells were compared with controls under an inverted microscope Leitz DM IL (Leica Microsystems GmbH, Wetzlar, Germany).

The cells were methanol-fixed and stained with 2% Giemsa-Romanovsky solution for 10 min. The effect of RAG at the selected concentrations was evaluated microscopically and compared with IC and SC.

Growth curves

Based on the results of the cytotoxicity test, the following RAG concentrations were chosen for evaluation of the proliferation activity of B-HNF-1 cells: 1×10^{-4}, 1×10^{-5} and 1×10^{-6} mol.l^{-1}. The cell suspension at the density of 1×10^5 cells per 1 ml was seeded into 35 mm Petri dishes in E-MEM with 10% FBS and gentamycin. Subsequently, RAG at the selected concentrations was added and cell cultures were placed in a CO_2 incubator. E-MEM was used as an IC and PBSa as an SC. The culture medium was aspired and the cells were rinsed with PBSa at 24 h intervals for five days. After enzymatic detachment, the cells at each tested concentration were counted using a haemocytometer and compared with the controls.

Micronucleus test

For the micronucleus test, the cell suspension of B-HNF-1 at a concentration of $1.1 \times 10^6.10$ ml^{-1} of E-MEM with 10% (w/v) FBS and gentamycin was seeded in 100 mm Petri dishes (Falcon). Subsequently, RAG in concentrations of 1×10^{-4}, 1×10^{-5} and 1×10^{-6} mol.l^{-1} was added. E-MEM was used as IC, physiological saline as SC and mytomycin C (MMC) at a final concentration of 3×10^7 mol.l^{-1} as a positive control (PC, Sigma, St. Louis, MO). Cells were maintained in a CO_2 incubator. According to growth curve courses, when the cells in IC and SC were in exponential phase of growth, Colcemide (Sigma) was added at a final concentration of 0.05 μg/ml. After three hours of incubation, slides for chromosomal analysis were prepared according to the conventional method. The slides were air-dried and stained with 2% Giemsa-Romanovsky solution for 10 min. Micronuclei were identified and counted in 3000 cells from second cell division, in three parallel karyological sections according to the previously published criteria (Countryman and Heddle, 1976).

Statistical analysis

To look for differences among groups, the numbers of micronuclei were analysed by post-hoc pairwise comparisons. Inferential statistics was made using Fisher's exact test for two independent proportions and "2 by k" χ^2 test for linear trend. Our statistical significance reporting criterion for contrasts between proportions was $P < 0.05$.

For statistical analysis we employed statistical software StatsDirect® version 2.3.7 (StatsDirect Sales, Sale, Cheshire, UK).

Results

Cell culture

B-HNF-1 cells have fibroblastoid morphology. The test result yielded 97% cell viability. The modal chromosome number of the cell line is 46, population doubling time is 24 hours, and the cells have a normal diploid male karyotype.

Cytotoxicity test

After 24 hours, B-HNF-1 cells exposed to RAG in the concentration range of 1×10^{-4} to 1×10^{-7} mol.l^{-1} were adhered to the bottom surface of the Petri dishes. No changes in the morphology of cytoplasm and nuclei when compared with control groups IC and SC (Fig. 2a) were observed. Cells treated with RAG at concentrations of 1×10^{-2} and 1×10^{-3} mol.l^{-1} remained in the suspension and were excluded from further testing.

After five days of cultivation, the cells exposed to RAG at a concentration of 1×10^{-6} and 1×10^{-7} mol.l^{-1} as well as the cells in IC and SC reached confluence (Fig. 2b). B-HNF-1 cells treated with RAG in a concentration of 1×10^{-5} mol/l growed up to a semi-confluent layer (Fig. 2c). The cells exposed to RAG at a concentration of 1×10^{-4} mol.l^{-1} formed an irregular network (Fig. 2d) and the cells had sporadically vacuolated cytoplasm. No changes in the morphology of nuclei were observed.

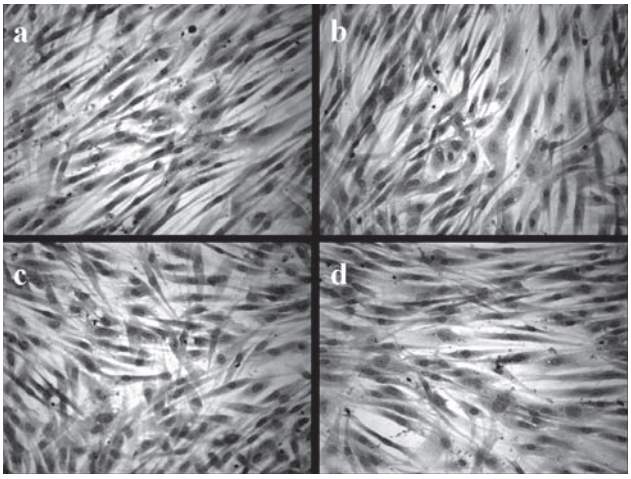

Fig. 2. **a)** Control cultures with confluent layers of fibroblast-like cells; **b)** Confluent layer of fibroblast-like cells exposed to RAG at a concentration of 1×10^{-6} mol.l^{-1}; **c)** Semi-confluent layer of fibroblast-like cells exposed to RAG at a concentration of 1×10^{-5} mol.l^{-1}; **d)** Irregularly sparse network of fibroblast-like cells after the exposure to RAG at a concentration of 1×10^{-4} mol.l^{-1}. (Magnification 400×)

Growth curves

Growth curves representing the results of proliferation of RAG-exposed and control cells are shown in Fig. 3. On the fourth day of cultivation, B-HNF-1 cells in IC, SC and RAG-exposed cells reached a plateau. The number of cells in IC and SC increased approximately 4-fold when compared with the inoculum. The number of cells exposed to RAG at concentrations of 1×10^{-6} and 1×10^{-5} mol.l^{-1} increased more than twice and almost twice, respectively. The number of cells treated with RAG at a concentration of 1×10^{-4} mol.l^{-1} increased up to 3×10^4 cells. The growth curve of these cells was characterized by a lag-phase on the fourth day of cultivation. Compared to the IC group, the proliferative activity of B-HNF-1 cells exposed to RAG at this concentration was inhibited. Hence, there is agreement in the results for both test systems.

Micronucleus test

Results from the micronucleus test are depicted in Table 1. The frequencies of micronuclei in the cells treated with RAG at the selected concentrations were close to the level of micronuclei in the IC and SC groups. No significant increase was detected in any of the RAG-treated group. In contrast, the frequency of micronuclei in the cells treated with MMC was significantly increased (P values less than 0.0013) when compared with all other groups.

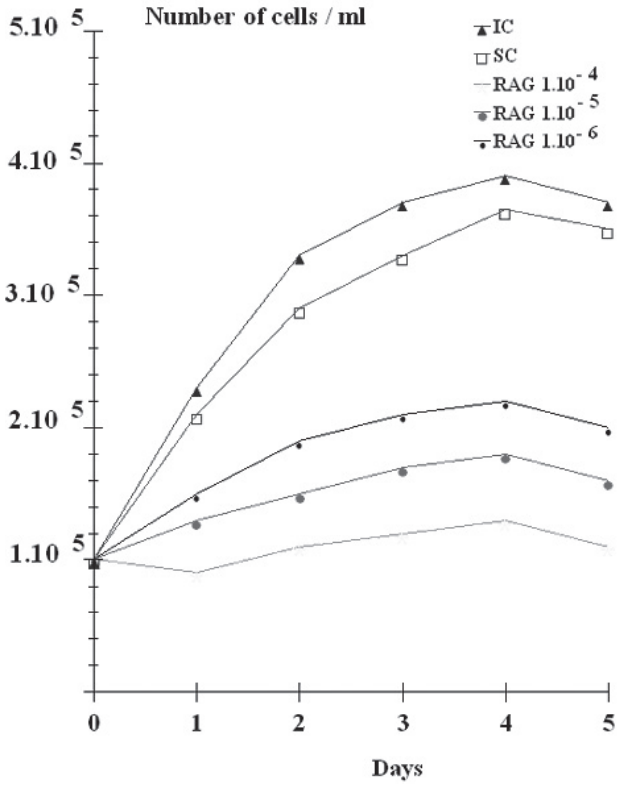

Fig. 3. Growth curves of IC, SC cells and cells treated with RAG at 1×10^{-4}, 1×10^{-5} and 1×10^{-6} mol.l^{-1}

Discussion

Aminoguanidine and its derivates have been shown to inhibit AGE formation due to their reaction with carbonyl products that are formed during the end phases of non-enzymatic protein glycation, both *in vitro* and *in vivo*. In our previous papers it was shown that AG and PAG in a concentration range of 1×10^{-4} to 1×10^{-6} mol.l^{-1} do not have a genotoxic effect (Vojtaššák et al., 2003; 2006). However, AG is unsuitable for administration to humans because it forms an adduct with pyridoxalphosphate (PLP), which is a coenzyme of transamination and decarboxylation reactions. The mentioned process results in deficiency of PLP and leads to a decreased activity of the enzymes that catalyse these reactions (Taguchi et al., 1998). Moreover, AG might also have prooxidative effects and, at high concentrations, it exhibited foetal toxicity in experimental animals (Sugyiama et al., 1986; Skamarauskas et al., 1996). In the light of potential toxicity/adverse effects of AG, the search for new inhibitors of protein glycation and glycoxidation with improved properties remains an important goal (Khalifah et al., 1999; Rahbar et al., 1999; Jain et al., 2001). Among other potential inhibitors, PAG was described to be a more efficient inhibitor of glycation of proteins and production of AGEs than AG (Taguchi et al., 1999; Miyoshi et al., 2002). Interesting results were also obtained in studies of the biological properties of RAG (Waczulíková et al., 2000a, b; 2002; Ziegelhöffer-Mihalovicová et al., 2003).

In this respect we have tested the genotoxic potential of RAG *in vitro*. For this experiment the B-HNF-1 cells were selected because human diploid cells, including neonatal fibroblasts, are generally accepted as a suitable biological model for *in vitro* cytotoxicity assessment (Tipton et al., 2003). There are also some other reasons for choosing human neonatal skin fibroblasts, i.e. the cells have favourable growth characteristics and are easy to handle. Growth curves showed an inhibitive effect of RAG on the proliferation of the exposed cells, and the inhibitory effect was directly proportional to the concentration of RAG. The effect was probably caused by antioxidation properties of RAG, which could be derived from its ability to form a redox system (Jakuš et al., 1999). Our analyses of the experimental data were therefore complemented by model considerations aimed at clarifying the mechanism through which RAG could inhibit the cell division. We took into account its polyfunctional physical and chemical properties, amphiphilic character and chelate forming. Amphiphilic properties follow from a balance between the free-base and ionic structure of the molecule at physiological pH. This steady-state made it possible that an interaction between RAG and homopolar or polar constituents of the cell membrane could occur, which might result in a modification of the cell membrane structure and its biological function (Waczulíková et al., 2000a; 2002). The mentioned finding might be beneficial in diabetes not only because of the improvement of impaired membrane functions controlled by membrane fluidity or antioxidative effect of RAG, but also as a result of protective function against potential liver damage (Liptáková et al., 2002).

Table 1. Frequency of micronuclei induced by resorcylidene aminoguanidine in cell line B-HNF-1

Tested substance	Concentration (mol.l^{-1})	No. of micronuclei	% of micronuclei	P value (against MMC)
Intact control	–	1	0.03	0.0003
Solvent control	–	1	0.03	0.0003
RAG	1×10^{-4}	2	0.07	0.0013
RAG	1×10^{-5}	2	0.07	0.0013
RAG	1×10^{-6}	2	0.07	0.0013
Positive control (MMC)	3×10^{-7}	24*	0.80*	–

The numbers in the PC (MMC) group were significantly different from those in IC as well as in the groups with the tested compound at all used concentrations. The groups with tested compound were not statistically different from the IC group, $P > 0.999$ (* the statistical significance reporting criterion for contrasts between proportions was $P < 0.05$).

It is also possible that some other kinds of chemical interactions on the cell membrane could take place, especially the interaction of RAG with the extracellular ligand-binding domain of some growth factor receptors with character of tyrosine kinases (RTK), G-proteins and cytokine receptors, which controls several signal pathways (Chang et al., 2004). Binding of RAG could significantly inhibit the downstream activation of the mitogen-activated protein kinases (MAPKs) signalling cascade in cooperation with other protein kinases. Protein kinase A (PKA) is a known regulator of the RAF-MAPK pathway starting the DNA synthesis, i.e. cell division – proliferation. RAG-evoked inhibition of cell proliferation, without any observable cytotoxic effect in the selected concentration range, may be of great interest for possible prospective application of RAG in the cancer treatment. Further detailed analysis of the cell cycle inhibition mechanism should be performed.

The results from MNT testing were evaluated according to the widely used criteria published by Countryman and Heddle (1976). RAG did not induce numeric or structural chromosomal aberrations. This is in agreement with the results obtained from the MNT test. Although it is recommend to add cytochalasin B to block cytokinesis but not nuclei division (Fenech, 2000), we did not use cytochalasin B because its application had no advantage for the cells (including fibroblasts), which underwent a normal cell cycle (Kalweit et al., 1999).

To summarize, the results of the presented study suggest that RAG in the used concentration range has an inhibitive effect on the proliferation of the exposed cells and, at the same time, does not display any cytotoxic and genotoxic effects on B-HNF-1 cells. These findings open a new field for further studies of substances with similar chemical structure and anti-glycation biological properties, as well as for investigation of the molecular mechanisms of their action.

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