ABSTRACT
Glycotoxins, products of Maillard’s reaction, are complex chemical substances. They are introduced by food or developed in the human organism. They have a negative effect on different structural and functional activities of important biological molecules in both cells and extracellular matrix. A model glycotoxin 3-hydroxy-1-methylpyridinium hydrochloride (HMP.HCl) was synthesized. HMP.HCl was derived from 3-hydroxypyridine by methylation with methyl iodide. Both the model glycotoxin and the starting 3-hydroxypyridine were investigated on cell cultures McCoy-Plovdiv and HepG-2 for cytotoxicity and genotoxicity activities. The test agents were used in a range of concentrations from 20 to 0.2 mg/ml. Quantity cytotoxicity assessment was determined by NR- and MTT assays and genotoxicity by Micronucleus assay. The higher cytotoxicity was determined for HMP.HCl compared to 3-hydroxypyridine, but no genotoxicity was determined without an exogenous activation. The water solution from the modifying compound HMP.HCl strongly decreased medium pH. Correlation was determined between medium pH and concentrations of test compound. We suggest that high acidity of culture medium causes the higher cell death.

Keywords: cell cultures, cytotoxicity, genotoxicity, glycotoxins, in vitro

Introduction
Thermal treatment of foods is a major method for their preparation. During this preparation take place multiple and complex processes known as Maillard reaction (7), responsible for chemical modification of proteins by carbohydrates or their degradation products(4). When Maillard reaction takes place in living systems it is referred to often as glycation. The final products of these reactions, named advanced glycation endproducts (AGE) normally are excreted. In patients with renal failure however they accumulate and could have negative impact on the organism (6). The harmful AGE are named glycotoxins, especially when they are formed in the foods during thermal treatment or storage.

In a previous work (1) was found that the compound 2-ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) is a newly identified AGE. It is a 3-hydroxypyridinium derivative of lysine and can be formed both in vitro and in vivo. The compound 3-hydroxy-1-methylpyridinium hydrochloride (HMP.HCl) is a simpler model of OP-lysine. The purpose of present study is the investigation of the biological activity of HMP.HCl

Materials and methods
Synthesis of 3-hydroxy-1-methylpyridinium hydrochloride (HMP.HCl).
The starting compounds 3-hydroxy pyridine (5.7 g, 60 mmols) and methyl iodide (3.72 ml, 60 mmols) were refluxed for one hour in 250 ml 1-propanol. The reaction product crystallized overnight at -32°C and then was dissolved in 30 ml water. Silver oxide (3.6 g, 15.4 mmols) was added to the solution in a centrifuge tube and stirred under sonication. The solid was separated by centrifugation and washed with 15 ml water. Silver oxide (3.3g, 14 mmols) was added to the combined water supernatants and stirred under sonication again. The solid was separated by centrifugation. The supernatant was filtered and extracted with 50 ml ethylacetate twice. The water phase was acidified with 30% HCl to pH 2 (white precipitate appeared immediately) and the precipitate was eliminated by filtration. The filtrate was concentrated under reduced pressure. After precipitation with acetone, the target compound 3-hydroxy-1-methylpyridinium chloride...
Cell cultures: Serum-free cell line McCoy-Plovdiv and hepatoma cell line HepG-2

McCoy-Plovdiv cells were cultivated in DMEM/Ham’s F-12 1:1 medium according to the procedures described in (3). Hepatoma cells were cultivated in medium DMEM supplemented with 10% fetal calf serum and subcultivating at each 6 days by tripsinization with 0.05% Trypsine and 0.02 EDTA (ethilen diamino tetra acetic acid). Cell cultures were incubating at 37°C, 5% CO2. Visual inspection and documentation of cell culture was made with microscope system Nikon Eclipse TC100.

Cytotoxicity Tests

Test agents were dissolved in growth media at the concentration 20 mg/ml and sterilized by membrane Millipore filter with pore size 0.22 µm. Substances were tested in the range of concentration 5000, 4500, 4000, 3500, 3000, 2500, 2000, 1500, 1000 µg/ml for 24 hours. Cytotoxicity effect was detected on McCoy-Plovdiv cell by NR test (2) and on HepG-2 cells by MTT test (8). Absorbance was detected on Multiskan MCC (Labsystem) on 410 and 540 nm to NR test and 540 nm and 620 nm to MTT test.

Genotoxicity test

Substances were tested for genotoxicity activities in the range of concentration 4000, 3500, 3000, 2500, 2000, 1500, 1000, 500, 250 µg/ml for 24 hours. Genotoxicity of the test agents was investigated in vitro on McCoy-Plovdiv cell line by Micronucleus test (BD Gentest Micronucleus Assay Kit from Becton Dickenson) Cytokinesis-blocked (binucleated) McCoy-Plovdiv cells were examined for the presence of micronuclei on Fluorescent microscopic system Nikon Eclipse 80i. The results were calculated following Khirsch-Volders et al (5). The microscopical analysis of the micronucleus test was performed by a single person.

Results and Discussion

The model glycoxotoxin HMP.HCl was studied for biological activity in vitro in wide range of concentrations. No survived cells were found after 24 h when the cell cultures McCoy-Plovdiv and Hep G-2 were treated with the highest concentrations of HMP.HCl (5000 and 4500 mg/ml). Microscopic evaluation showed changes in the cell morphology (Fig. 1B and 1D). It can be seen that there is drastic destruction of the cell monolayer in comparison to the control, non treated cultures (Fig. 1A and 1C). The damaged cells are rounded and squeezed. Part of them are still attached to the wall but they are not vital, which was confirmed later by the vitality tests. At concentration 4000 µg/ml it was found significant increase of the percentage of the survived cells compared to the control ones at 65.3% and 71.8% correspondingly for McCoy-Plovdiv and HepG-2. The percentage of the survived cells increases in parallel with the decrease of the concentration of 3-hydroxypyridine and for 2000 µg/ml it is more than 90% according to both tests. For concentrations of 3-hydroxypyridine lower than 3500 µg/ml one could say that the cells are vital but the cell growth is inhibited and there are almost no dividing cells. Based on the vitality tests obtained, the IC50 values for McCoy-Plovdiv and HepG-2 were calculated: 4117.2 µg/ml and 4158.4 µg/ml correspondingly. A weaker cytotoxic effect was found for 3-hydroxymethyl pyridine. In both cultures there were a lot of survived cells at concentration 5000 µg/ml – 46.7% for McCoy-Plovdiv and 30.7% for HepG-2. The IC50 values are 4791.1 µg/ml and 4563.3 µg/ml correspondingly. The correlation concentration effect is valid also for 3-hydroxymethyl pyridine. The cytotoxic effect of HMP.HCl and 3-hydroxypyridine was shown using two different tests and two different cell lines. For the former it was found that it increases significantly the acidity of the medium for cell cultivation. The pH values show direct correlation with the concentration of HMP.HCl. In the range from 3000 µg/ml to 1000 µg/ml pH changes from 5.50 to 7.05 and each decrease of the concentration with 200 µg/ml results in increase of pH with 0.15. The growth medium with pH 7.50 in which was dissolved HMP.HCl in concentration 10 mg/ml decreased pH to 4.65, while 3-hydroxypyridine decreases the pH to 7.10 even at concentration of 20 mg/ml. These results show that in the case of 3-hydroxopyridine the cells were treated under normal physiological values of pH of the medium while in the case of HMP.HCl pH is much lower than the recommended values for the growth medium with pH in the range 7.20 - 7.40. The acidity factor additionally masks the true activity of HMP.HCl. The results obtained allow us to define HMP.HCl as a compound with high cytotoxicity which was proven for both cell lines by two different tests. As a result of the chemical modification this glycoxotoxin transforms into a compound with strong acidic nature. HMP.HCl and 3-hydroxypyridine were studied for direct genotoxic effect using in vitro Micronucleus assay. Both compounds do not cause clastogenic or aneugenic effects on the cell line McCoy-Plovdiv. No studies were performed to prove that they are proclastogenic and turn to genotoxic after a preliminary metabolic transformation.
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REFERENCES

Fig. 1. Morphological effects on cells from cell lines McCoy-Plovdiv and HepG-2 of glycotoxin HMP.HCl treatment for 24 hours. A. Control, non treated Hep G-2 cells. B. Hep G-2 cells treated with 5000 µg/ml HMP.HCl. C. Control, non treated McCoy-Plovdiv cells. D. McCoy-Plovdiv cells treated with 5000 µg/ml HMP.HCl. Bar scale: 1 cm = 60 µm.