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INVESTIGATIONS OF GENOTOXIC ACTIVITY OF ANTIMICROBIAL/ ANTIVIRAL AGENT FS-1 IN HUMAN LYMPHOCYTES AND TUMOR CELL

A very promising antiviral and antimicrobial agent FS-1 was studied for its ability to induce DNA damage and micronuclei in human tumor cell lines HeLa and Caco-2 at concentrations of 200, 500 and 1000 μg/ml without exogenous metabolic activation. The compound was additionally tested for DNA damaging ability in human lymphocytes at concentrations of 200, 400 and 800 μg/ml. Neither DNA damage nor micronucleus formation was observed after treatment of all types of cells with FS-1. Based on these results, FS-1 can be further studied for its safety to humans for potential application in clinical medicine as an antimicrobial/antiviral drug.

Introduction. Recently very effective drug against a number of strains of microbes and viruses was invented and patented in Kazakhstan (KZ Patent № 15116). FS-1 is a complex of iodine with synthesized polysaccharides, i.e., it is an iodophore, a combination of iodine and a solubilizing agent that releases free iodine when diluted with water. Iodophores possess quick microbial action against a wide variety of antimicroorganisms, e.g., bacteria, viruses, fungi and protozoa [1].

FS-1 is very potent in veterinary medicine against many infectious factors of microbial and viral origin. Molecular mass of the compound is 32 500. Because of its low toxicity in rodents (i.e., 25 ml/kg is well tolerated dose, containing 19.9 g/l of active ingredients – unpublished data of Scientific Center for Anti-Infectious Drugs, Almaty, Kazakhstan) it is possible to use FS-1 in the clinical medicine as microbicide, if it meets all international safety criteria. These include, in the first turn, the studies on mutagenicity of the compound.

The compound has been tested for its mutagenicity in the *Salmonella*/microsome (Ames) assay in five strains of microbes (TA 98, TA 100, TA 102, TA 1535, TA 1538) with and without metabolic activation at doses up to 500 μl/plate (which was the limit of bacterial toxicity). Completely negative results were obtained (Prof. S. Knasmüller, Medical University of Vienna, personal communication).

DNA-damaging and micronucleus (MN) inducing activity of FS-1 in mammalian cells in *in vitro* systems is unknown. The aim of this work was to evaluate DNA-damaging activity of FS-1 in human tumor cell lines and lymphocytes, and also MN inducing ability in human tumor cell lines.

Material and methods. *Chemicals.* FS-1 was produced in RSOE «Anti-Infectious Drugs», Almaty, Kazakhstan. All other chemicals used in experiments were produced by Sigma-Aldrich (St. Louis, USA). FS-1 is an aqueous solution of iodine–lithium inclusion complex with low molecular weight α-dextrin and polyvinyl alcohol. FS-1 contains also potassium iodide, lithium and sodium chlorides. The compound has almost the same composition as iodine-lithium-alpha-dextrin used in some countries as a potent antimicrobial/antiviral agent [2].

Cells. HeLa (human cervix carcinoma) and Caco-2 (epithelial colorectal adenocarcinoma) cells were obtained from Laboratory U 322 INSERM «Retrovirus et Maladies Associées»,

Marseilles, France, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated foetal bovine serum and 100 U/ml penicillin/streptomycin. Cells were kept at 37 °C in humidified 5 % CO_2 and 95 % air and were allowed to grow in complete medium (containing serum) for 24 h before the start of experiments. The experiments were conducted in flasks containing 5 ml of medium with $2 \cdot 10^6$ HeLa or Caco-2 cells at concentrations of FS-1 100, 200, 500, 1000 and 2000 μg/ml (incubation time – 24 h). Viability of cells was assessed by means of trypan blue dye exclusion technique, i.e., the number of viable cells was calculated as described earlier [3]. Cells were identified as viable (bright yellow), compromised (bluish), and dead (completely blue). Only bright yellow cells were considered. Since 2000 μg/ml was highly toxic for both HeLa and Caco-2 cells (90 % dead cells), and 1000 μg/ml of FS-1 induced death only in ca. 30 % cell, the compound was tested at doses of 200, 500 and 1000 μg/ml.

Blood samples were obtained by venous puncture from two male and two female healthy donors-volunteers (non-smokers, 21–25 years old). Lymphocytes were separated by centrifugation (20 min, 2000 r.p.m., at room temperature) in equal amounts of Lymphoprep («Nycomed», Norway), washed in phosphate-buffered saline. One million lymphocytes were incubated for 24 h with various concentrations of FS-1 (1000, 800, 400 and 200 μg/ml). The first concentration was very toxic for lymphocytes. Eight hundred μg/ml of the compound decreased the viability of cells to ca. 70 %, and this concentration was chosen as an upper limit of the compound concentration in the study. Other tested concentrations of FS-1 used were 400 and 200 μg/ml. In the first experiment with lymphocytes freshly isolated cells were used. In the second experiment, conducted later, lymphocytes stored at –80 °C were used.

Comet assay. Comet (single cell gel electrophoresis) assay was carried out as described in our recent papers [4]. Cells were stained with ethidium bromide. Cells were analyzed visually by means of LM-6 microscope (USSR). One hundred cells on each slide (3 slides per each culture, 900 cells per experimental point or, in other words, per concentration) were scored as belonging to one of five classes of comets according to tail intensity and given a value of 0, 1, 2, 3 and 4 (from undamaged 0 to maximally damaged 4). Thus, the total score for 100 comets (called arbitrary unit) ranges from 0 (all undamaged cells to 400 (all maximally damaged) [4]. Some examples of comets are presented in Fig. 1–4.

A mean value of arbitrary units obtained from each culture was considered as an independent variable. Methyl methanesulfonate (MMS) was used as a positive control in all experiments (30.0 μg/ml, 3 h incubation) because of its high genotoxic potency. As negative control phosphate buffered saline (PBS) was used. Two independent experiments were carried out.

Micronucleus (MN) assay. The assay was conducted as described earlier [3]. After the growing in the complete medium (containing serum) for 18 h, HeLa and Caco-2 cells were incubated 24 h with FS-1 at concentrations of 1000, 500 and 100 μg/ml. Cisplatin (1.5 μg/ml) was used as a positive control. As a negative control PBS was used. Three simultaneous cultures were prepared, and the experiments were carried out two times. Consequently, microscopic slides with cells were prepared for analysis.

Two thousand cells were studied from each slide for cytogenetic alterations under microscope, 6000 per concentration (MBI-6).

*Statistical analysis***.** Mann-Whitney U-test was applied to calculate differences between the groups. The mean value of DNA damage which was obtained from each culture and expressed in arbitrary units, was considered as an independent variable. Three numbers obtained per concentration in the first experiment were compared with analogues data from the second experiment. In no case a significant difference was obtained. So, 6 variables of each experimental point were compared with 6 variables obtained from positive and negative controls, respectively.

In the MN assay the independent variables were number of cells with MN and total number of MN, expressed in pro mille. Similarly, no significant differences were found between the data obtained from both experiments. The data were evaluated statistically as previous set of results.

The differences between three experimental groups and negative controls were evaluated in all

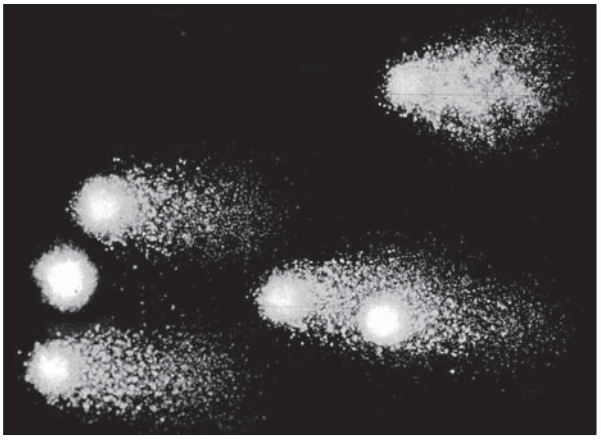

Fig. 1. Various grades of comets in human lymphocytes. On the left can be seen a non-damaged cell (0 comet score) located between 2 severely damaged cells (comet score 4)

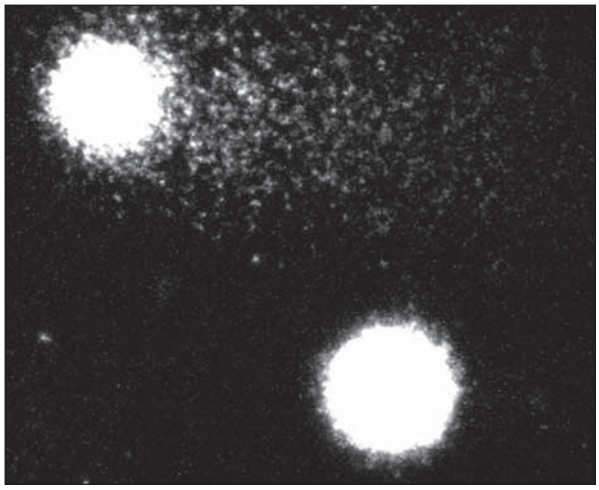

Fig. 2. Various grades of comets in human lymphocytes. Undamagent cell is located below a cell with comet grate 2 (above)

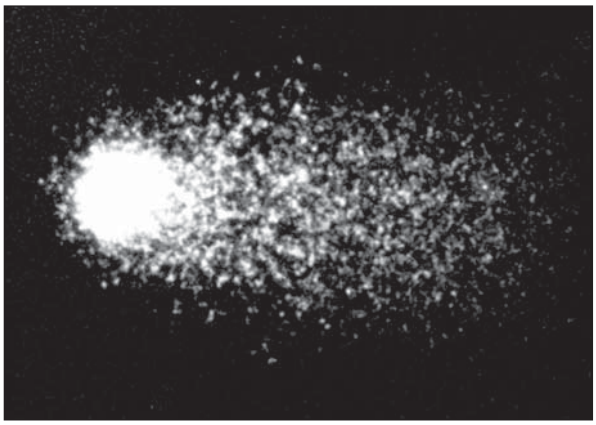

Fig. 3. Lymphocyte with comet score of 4 (severely damaged)

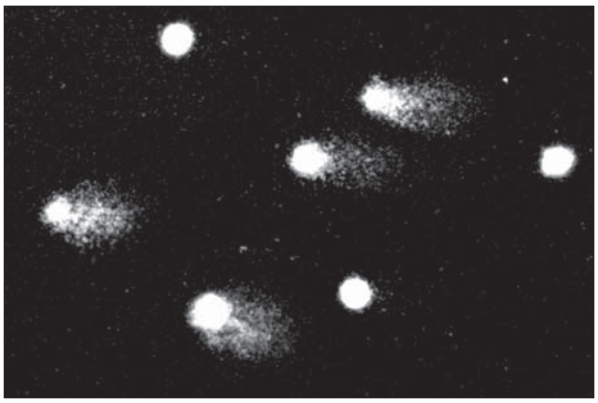

Fig. 4. HeLa cells with comets of various grades

cases by means of Kruskal-Wallis test. Also, in experiments with human lymphocytes Kruskal-Wallis test was applied to calculate inter-individual and gender differences.

Results and discussion. Iodophores such as povidone–iodine are broad-spectrum microbicides with activity against bacteria, viruses, fungi and protozoa. They consist of elementary or ionized iodine bound to polymer carriers (such as poly-1-vinyl-2-pyrrolidine and dextrins), which increase solubility and provide a reservoir of iodine [1]. Due to the oxidizing effects of free iodine on key groups of proteins, nucleotides, fatty acids and the subsequent non-specific mechanism of cell killing, iodophores are used as potent microbicides [1, 2].

Taking this into account, it is of interest to study FS-1 in the short-term genotoxicity assays. Since the compound is supposed to be used in clinical medicine, it is mandatory and necessary to evaluate its possible genotoxic activity [5].

We studied the activity of FS-1 in two widely used short-term genotoxicity assays, i.e., in the comet and MN assays, which are recommended for testing of various compounds [6–9]. Human lymphocytes as well as HeLa and Caco-2 human cancer cell lines are the most frequently used cell for various kinds of investigations, including DNA damage and MN studies [6–9].

In human HeLa and Caco-2 tumor cells, FS-1 at a dose of 1000 μg/ml induced about 30 % cell death. This concentration was used as an upper limit in our experiments since in the guidelines for MN assays *in vitro* [6] concentrations which induce about 30–50 % cell toxicity are recom-

mended as an upper limit. Also in the comet assay 1000 μg/ml was used as a highest concentration because cytotoxic action of the chemical studied in the comet assay should not exceed 30 % [9].

The results of the experiments on potential DNA-damaging effect of FS-1 in two tumor cell lines are presented in Table 1. It can be seen that no genotoxic effect was found after incubation of cells with FS-1 for 24 h in both cell lines. The positive control induced substantial DNA damage in all experiments. The background DNA damage level in both cell lines was in the range of previous published results [3, 4]. The differences between the three experimental groups and the negative controls were estimated by means of Kruskal-Wallis test. No statistically significant difference was obtained.

Human lymphocytes were more sensitive to acute toxic action of FS-1 than cancer cells since the concentration of 1000 μg/ml was highly toxic. Neither sex nor inter-individual differences were obtained since no statistically significant difference was found when Kruskal-Wallis test was applied. Because of this the data were pooled.

Table 1

DNA-damaging activity of FS-1 in human tumor cell lines HeLa and Caco-2 (visual score, mean of two independent experiments)

Compound and concentration, μg/ml	Percent of viable cells	DNA damage (arbitrary units, mean ± SE)
	HeLa	
FS-1 (1000)	69.3 ± 1.4 *	16.4 ± 1.1
FS-1 (500)	91.2 ± 0.9	14.0 ± 1.0
FS-1 (200)	93.8 ± 1.1	11.5 ± 0.9
MMS (30)	86.3 ± 1.9 **	45.7 ± 2.0 *
PBS	96.5 ± 1.1	10.0 ± 0.9
	Caco-2	
FS-1 (1000)	71.8 ± 1.78 *	14.7 ± 1.5
FS-1 (500)	91.2 ± 1.14	12.7 ± 1.0
FS-1 (200)	94.8 ± 1.1	11.8 ± 1.4
MMS (30)	82.8 ± 2.2 **	40.4 ± 2.3 *
PBS	95.5 ± 1.1	9.7 ± 1.2

Note. MMS – methylmethane sulfonate; PBS – phosphate buffered saline; the difference between negative control and FS-1 treated groups was not statistically significant (Kruskal-Wallis test). *p < 0.002, **p < 0.05, Mann-Whitney U-test.

Table 2

DNA-damaging activity of FS-1 in human lymphocytes (pooled data obtained from 2 male and 2 female donors, visual score, mean of two independent experiments)

Compound and concentration, μg/ml	Percent of viable cells	DNA damage (arbitrary units, mean ± SE)
FS-1 (200)	93.6 ± 0.6	10.0 ± 0.5
FS-1 (400)	93.5 ± 0.6	10.0 ± 0.5
FS-1 (800)	75.7 ± 0.9 *	9.4 ± 0.5
MMS (30)	86.3 ± 1.0	47.4 ± 0.9 *
PBS	93.5± 0.5	4.9 ± 0.3

Note. The difference between negative control and FS-1 treated groups was not statistically significant (p > 0.05, Kruskal-Wallis test). *p < 0.002, Mann-Whitney U-test.

Table 3

Micronucleus inducing activity of FS-1 in human cancer cell lines HeLa and Caco-2 (mean of two independent experiments)

Compound and concentration, μg/ml	Percent of viable cells	Cells with MN, ‰	Total number of MN, ‰
	HeLa		
FS-1			
1000	72.0 ± 1.7 *	4.2 ± 0.6	4.4 ± 0.7
500	90.3 ± 1.3	3.0 ± 0.7	3.3 ± 0.8
200	94.5 ± 0.9	3.6 ± 0.4	4.0 ± 0.4
Cisplatin			
1.5	81.8 ± 1.6 **	36.6 ± 1.7 *	42.4 ± 1.9 *
Negative control (PBS)	94.5 ± 1.0	3.2 ± 0.3	3.6 ± 0.3
	Caco-2		
FS-1			
1000	74.3 ± 1.6 *	3.2 ± 0.5	3.5 ± 0.5
500	90.3 ± 1.3	3.2 ± 0.4	3.2 ± 0.4
200	93.2 ± 2.0	2.6 ± 0.4	2.6 ± 0.4
Cisplatin			
1.5	82.5 ± 1.8 **	29.2 ±1.6*	34.0 ± 1.7*
Negative control (PBS)	97.0 ± 1.0	2.6 ±0.2	2.8 ± 0.3

Note. The difference between negative control and FS-1 treated groups was not statistically significant (p ⩾ 0.05, Kruskal-Wallis test). *p < 0.002, **p < 0.05, Mann-Whitney U-test.

The results of the comet assay with human lymphocytes are presented in Table 2. As can be seen, no significant changes compared with the nega-

tive control were observed. In addition, the differences between the three experimental groups and the negative controls were estimated by means of Kruskal-Wallis test. No statistically significant difference was obtained.

Hence, FS-1 showed no DNA-damaging activity in the comet assay in human lymphocytes and tumor cell lines HeLa and Caco-2.

The results of the MN assay are presented in Table 3. It can be noted that FS-1 did not induce an increase either in the number of cells with MN or total number of MN in both cell lines. Background levels of MN in untreated cells and cisplatin-induced MN frequencies were similar to our previous reported results [3].

HeLa cells lack the ability to metabolize mutagens/carcinogens because of absence of phase I enzymes whereas Caco-2 possesses ability for metabolic activation of xenobiotics by means of cytochromes CYP1A1, CYP1A2 and CYP1B1 [10, 11]. Human lymphocytes possess cytochrome P450 2E1 but it is not enough for metabolic activation of all xenobiotics [12]. The absence of genotoxic activity of FS-1 in lymphocytes and Caco-2 cells which possess more or less drug metabolism phase I enzymes, and also in HeLa cells suggest that either the compound has no ability to be transformed into biologically active metabolites or after the transformation of the compound metabolites have equal genotoxic potential with the parent compound. Our data support completely negative results in the Salmonella/microsome assay in five strains of microbes both with and without metabolic activation with S9 mix (Prof. Knasmüller, personal communication).

Since the tested compound is iodophore, it would be of interest to stress that widely used representative of this kind of preparations, povidone-iodine, was found non-genotoxic in some *in vitro* mutagenicity tests in bacteria and mammalian cells as well in mouse bone marrow MN assay [13–15].

Hence, the results obtained in the present set of experiments are very important for the next level of genotoxicity studies of FS-1 which can be used in clinical medicine (especially as topical microbicidal/antiviral agent) after routine testing of safety for humans.

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ИССЛЕДОВАНИЯ ГЕНОТОКСИЧЕСКОЙ АКТИВНОСТИ АНТИБАКТЕРИАЛЬНОГО/ПРОТИВОВИРУСНОГО ПРЕПАРАТА ФС-1 В ЛИМФОЦИТАХ И КЛЕТКАХ ОПУХОЛИ ЧЕЛОВЕКА

Исследовали способность перспективного антивирусного и антибактериального соединения ФС-1 вызывать повреждения ДНК и микроядра в клеточных линиях опухоли человека HeLa и Caco-2 при концентрациях 200, 500 и 1000 мкг/мл без экзогенной метаболической активации. Соединение было дополнительно проверено на ДНК-повреждающую способность в лимфоцитах человека при концентрациях 200, 400 и 800 мкг/мл. Ни повреждения ДНК, ни формирования микроядер не наблюдалось после обработки всех типов клеток ФС-1. На основании этих результатов, ФС-1 может быть далее изучен на предмет безопасности для возможного применения в клинической медицине как антибактериального/противовирусного препарата.

А. Нерсесян, А. Ільїн, М. Кулманов

ДОСЛІДЖЕННЯ ГЕНОТОКСИЧНОЇ АКТИВНОСТІ АНТИБАКТЕРІАЛЬНОГО/ПРОТИВІРУСНОГО ПРЕПАРАТУ ФС-1 В ЛІМФОЦИТАХ ТА КЛІТИНАХ ПУХЛИНИ ЛЮДИНИ

Досліджували здатність перспективного противірусного та антибактеріального препарату ФС-1 викликати пошкодження ДНК та мікроядра в клітинних лініях пухлини людини HeLa і Caco-2 при концентраціях 200, 500 та 1000 мкг/мл без екзогенної метаболічної активації. Препарат був додатково перевірений на ДНК-пошкоджуючу здатність в лімфоцитах людини при концентраціях 200, 400, 800 мкг/мл. Ні пошкодження ДНК, ні формування мікроядер не спостерігалось після обробки всіх типів клітин ФС-1. Базуючись на цих результатах, ФС-1 може далі вивчатись на предмет безпеки для потенційного застосування в клінічній медицині як антибактеріального/противірусного препарату.

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