

Investigations of genotoxic activity of the antimicrobial/antiviral agent FS-1 in rodents by means of the micronucleus and the comet assays

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SUMMARY

Background: FS-1 is a complex of iodine and synthesized polysaccharides and it is very effective against a number of microbe and virus strains. The aim of the study was to evaluate possible genotoxic properties of FS-1.

Methods: The compound was studied in rat and mouse bone marrow micronucleus (MN) assay and the comet assay in murine peripheral blood leukocytes, hepatocytes, and kidney cells. Two treatment protocols were applied, namely acute and subacute ones. In the first protocol, the compound was administered orally once and in subacute treatment two times, 24 h apart. The animals were sacrificed 24 h after the last treatment, and appropriate cells were used to assess DNA damage and MN induction.

Results: In none of the tests (MN and comet assays) significant increase compared with respective negative controls was observed.

Conclusion: The fact that the compound neither induces DNA damage in various organs of mice nor is effective in the induction of MN in bone marrow cells of rats and mice is important for future genotoxicity studies of FS-1, which can be used in clinical medicine after additional testing of safety for humans.

Key words: Mice; Rats; Comet Assay; Micronucleus Tests; Anti-Infective Agents; Drug Toxicity; DNA Damage

INTRODUCTION

A very effective drug against a number of strains of microbes and viruses has recently been invented and patented (KZ Patent No. 15116) in Kazakhstan. FS-1 is a complex of iodine with synthesized polysaccharides; i.e., it is an iodophor, a combination of iodine and a solubilizing agent that releases free iodine when diluted with water. Iodophors possess a quick microbicide action against a wide variety of microorganisms such as bacteria, viruses, fungi, and protozoa (1).

FS-1 is very potent in veterinary medicine against many infectious factors of microbial and viral origin. Because of its low toxicity in rodents [i.e., 25 ml/kg is the maximum tolerated dose (MTD) for both mice and rats; Ilin, unpublished data] it is possible to use FS-1 in the clinical medicine as microbicide and antiviral agent, if it meets all international safety criteria. These include, in the first turn, the studies on genotoxicity of the compound. *In vitro* studies of cell cultures infected by HIV and H5N1 viruses have shown that povidone-iodine has an antiviral action, while the cell hosts survive without being affected (2). Some recent publications suggest that when common preparations of iodine are used, significant quantities of the lipophilic form are delivered to cells of the reticuloendothelial system. Based on the relative distribution of iodine found in plasma and lymph (i.e. as a percentage of the dose administered), concentrations known to be effective *in vitro* could be readily achieved by oral ingestion (3-5).

IL α D (iodine-lithium- α -dextrin) is an aqueous solution of iodine-lithium inclusion complex with low molecular weight α -dextrin and polyvinyl alcohol (5). Recently, clinical efficacy, tolerability, and safety of IL α D monotherapy have been evaluated in HIV-infected patients by intravenous administration of 12 infusions in 4 cycles. IL α D therapy contributes to

anti-HIV and anti-inflammatory effects, resolution of dermatological and neurological pathology, and it dramatically improves the quality of life reflecting on enhanced treatment adherence. IL α D appears to be safe and prospective as an adjuvant therapy for bacterial and viral infections, including HIV/AIDS, hypothyroid, autoimmune, and inflammatory diseases by controlling pathogen production from infected cells, immune response, inflammation and metabolism (5).

FS-1 is a compound structurally very similar to IL α D. The compound is planned to be applied in a small clinical trial in HIV-infected patients. Before the trial, the safety of the compound should be proved.

FS-1 was tested for its mutagenicity in the *Salmonella*/microsome 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 μ l/plate (which was the limit of bacterial toxicity). Completely negative results were obtained (Prof. S. Knasmüller, Medical University of Vienna, a manuscript in preparation).

FS-1 was studied for its ability to induce DNA damage and micronuclei (MN) frequency in human tumor cell lines HeLa and Caco-2 and mouse lymphoma cell line L5178Y at concentrations of 200, 500, and 1000 μ g/ml without exogenous metabolic activation (6, 7). The compound was additionally tested for DNA damaging ability in human lymphocytes at concentrations of 200, 400 and 800 μ g/ml. Neither DNA damage nor MN formation was observed after treatment of all types of cells with FS-1 (6, 7). DNA-damaging and MN inducing activity of FS-1 in mammalian cells in *in vivo* systems is unknown. The aim of this work was to evaluate DNA-damaging activity of FS-1 in leucocytes, hepatocytes and kidney cells, and MN induction in bone marrow cells of mice. Genotoxicity tests are

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useful tool in the investigation of possible harmful effects of drugs (i.e. carcinogenicity) in the treatment of humans. Brambilla et al. (8) reviewed the literature data concerning DNA-damaging activity of 146 pharmaceuticals widely used in modern medicine and showed 63.1% concordance in the case of DNA strand breaks and carcinogenicity of the drugs.

MATERIALS 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- α -dextrin used in some countries as a potent antimicrobial/antiviral agent (5).

Animals and dosing

Experiments were carried out on male Swiss albino mice, weighing 23–25 g and Wistar male rats, weighing 120 g. The animals were acquired from the animal house of the Institute of Fine Organic Chemistry, Yerevan, Armenia and kept in polyethylene boxes. Food and water were available *ad libitum*. The mice were divided into experimental groups per 6 animals in each. All mice were administered with single or double doses of FS-1 at volumes of 10 ml/kg, 5.0 ml/kg, and 2.5 ml/kg BW by gavage. The negative control group received physiological saline at volume of 10 ml/kg twice. The positive control group received a single intraperitoneal (IP) injection of ethylnitrosourea (N-nitroso-N-ethylurea, ENU) at dose of 50 mg/kg. The acute and subacute protocols of MN assay in bone marrow of rodents introduced by Gene-Tox Program were applied (9). These protocols were successfully used in our previous experimental studies (10).

The rats were divided into experimental groups per 5 animals in each. The rats were administered with single or double doses of FS-1 at volumes close to MTD and 1/2 of MTD (20 and 10 ml/kg) by gavage. As positive control, cyclophosphamide at dose of 25 mg/kg IP was used dissolved in distilled water immediately before use. The negative control group received physiological saline at volume of 20 ml/kg twice.

In both acute and subacute treatment groups, the rodents were sacrificed 24 h after the last treatment. Mice used in this study were sacrificed by decapitation. Rats were sacrificed with CO₂.

Micronucleus assay

Bone marrow from both femurs of mice was flushed out onto slides using 0.2 mL calf fetal serum, then fixed with chilled 80% methanol and stained with May-Gruenwald/Giemsa (9). For the analysis of the cells with MN, 2000 polychromatic erythrocytes (PCE) per animal were scored to determine the mutagenic property of the compound. To detect possible cytotoxic effects, the PCE:NCE (normochromatic erythrocytes) ratio in 1000 erythrocytes/animal was calculated (9). The cells were blindly scored using a light microscope at 1000x magnification (Nikon Eclipse E200). The slides from bone marrow of rats were prepared as described for mice but the slides were stained with acridine orange to avoid false positive

results (8). The slides were studied under fluorescent microscope Nikon Eclipse E400 (Japan).

Comet assay

The comet assay (single-cell gel electrophoresis, SCGE) was carried out according to the method described by Sasaki et al. (11) and Cordelli et al. (12) using the same animals mentioned in the previous section. After the mice were sacrificed, 5 μ L of peripheral blood were collected from each animal. Livers and kidneys of mice were minced, suspended at a concentration of 1 g/ml in chilled homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na₂EDTA, homogenized gently using a Potter-type homogenizer at 500–800 rpm set in ice, and then centrifuged nuclei were used for the comet assay. 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 (12). Cells were identified as viable (bright yellow), compromised (bluish), and dead (completely blue), and only bright yellow cells were considered. Cells were further assessed for DNA damaging activity only in case of their viability \geq 80%. A 5- μ L aliquot of hepatocytes, kidney cells or blood from each animal was mixed with 120 μ L 0.5% low melting point agarose at 37°C, and rapidly spread onto two microscope slides per animal, pre-coated with 1.5% normal melting point agarose. The slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL distilled water and 1% sodium lauryl sarcosine), plus 1 mL Triton X-100 and 10 mL dimethylsulfoxide. The slides, which were protected from light, were allowed to stand at 4°C for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH-1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4°C for 20 min prior to electrophoresis, to allow DNA unwinding. The electrophoresis run was carried out in an ice bath (4°C) for 20 min at 300 mA and 25 V. The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature. For the staining, the slides were covered with 30 μ L ethidium bromide solution and coverslipped. The material was evaluated immediately at 400x magnification, using a fluorescence microscope (Olympus BX 50).

For each experimental point, three slides were prepared in parallel and from each, 50 cells were analyzed by a blinded observer with a computer aided image analysis system (Comet Assay IV Perceptive Instruments Ltd., Haverhill, UK). As endpoint, the percentage of DNA in tail was recorded as the most important in the comet assay (13).

Ethics

The Ethics Committee of the National Center of Oncology, Yerevan, Armenia approved the present study (protocol number 12-1/07).

Statistical analysis

GraphPad Software, Inc. (La Jolla, CA, USA) was used to calculate the difference between the groups. For statistical evaluation of the results, obtained Mann-Whitney U-test and Kruskal-Wallis with Dunn's multiple comparison tests were applied.

RESULTS

Since the compound is supposed to be used in clinical medicine, it is mandatory to evaluate its possible genotoxic potency (14). We studied the activity of FS-1 in two widely used short-term genotoxicity assays, i.e., in the comet and MN assays in rats and mice bone marrow cells recommended for testing of new synthesized compounds which can be used as pharmaceuticals (15, 16).

Combining these two assays with differences in sensitivity, endpoints measured and the type of data generated significantly improves upon the current standard capabilities for detecting genotoxicity without requiring additional animals (17).

The results of the MN experiments are presented in Tables 1 and 2.

Table 1. Micronucleus-inducing effect of acute and subacute treatments of mice with FS-1 in bone marrow polychromatic erythrocytes (means \pm SD)

Groups of mice with various treatments (dose in ml/kg or mg/kg)	Treatment	Number of PCE with MN ($\%$)	Number of MN ($\%$)	Content of PCE among erythrocytes ($\%$)
FS-1 (10)	Acute	1.08 \pm 0.58	1.33 \pm 0.41	51.17 \pm 1.52
	Subacute	1.58 \pm 0.38	2.00 \pm 0.55	50.50 \pm 1.36
FS-1 (5)	Acute	1.25 \pm 0.52	1.42 \pm 0.58	51.83 \pm 2.32
	Subacute	1.50 \pm 0.45	1.92 \pm 0.38	51.50 \pm 1.92
FS-1 (2.5)	Acute	1.67 \pm 1.17	2.00 \pm 1.27	50.67 \pm 1.51
	Subacute	2.00 \pm 1.10	2.83 \pm 1.37	54.67 \pm 1.21
Positive control ENU (50)	Acute	14.92 \pm 2.78*	16.5 \pm 2.49*	49.50 \pm 1.50
	Subacute	17.25 \pm 3.48*	19.25 \pm 3.50*	48.80 \pm 1.98
Negative control (10)	Subacute	1.25 \pm 0.27	1.42 \pm 0.38	54.83 \pm 2.07

* $p < 0.001$ (Mann-Whitney U-test and Kruskal-Wallis test with Dunn's multiple comparison test) compared with respective negative control; the doses of treatment were ml/kg except ENU (ethylnitrosourea) – mg/kg; in all groups were 6 rodents

Table 2. Micronucleus-inducing effect of acute and subacute treatments of male Wistar rats with FS-1 in bone marrow polychromatic erythrocytes (means \pm SD)

Groups of mice with various treatments (dose in ml/kg or mg/kg)	Treatment	Number of PCE with MN ($\%$)	Number of MN ($\%$)	Content of PCE among erythrocytes ($\%$)
FS-1 (20)	Acute	3.40 \pm 0.74	3.40 \pm 0.74	53.40 \pm 2.88
	Subacute	3.20 \pm 0.91	3.20 \pm 0.91	52.60 \pm 2.61
FS-1 (10)	Acute	3.60 \pm 0.93	3.50 \pm 0.93	53.20 \pm 2.30
	Subacute	4.40 \pm 1.27	4.80 \pm 1.29	53.00 \pm 2.45
Positive control CPA (25)	Acute	19.20 \pm 1.62*	21.60 \pm 2.49*	51.00 \pm 1.58
	Subacute	24.20 \pm 2.17*	26.40 \pm 3.14*	50.20 \pm 1.48
Negative control (20)	Subacute	2.20 \pm 0.57	2.20 \pm 0.57	54.40 \pm 2.45

* $p < 0.001$ (Mann-Whitney U-test and Kruskal-Wallis test with Dunn's multiple comparison test) compared with respective negative control; the doses of treatment were ml/kg except CPA (cyclophosphamide) – mg/kg; in all groups were 5 rodents

As can be seen, no increase of PCEs with MN and total number of MN were found in bone marrow of mice and rats treated with FS-1 according to both protocols. In addition, no changes were found in the content of

PCE in bone marrow after FS-1 treatment compared with the respective negative controls. These observations showed that neither genotoxic effect (both clastogenic and aneugenic) nor acute bone marrow toxicity was induced by FS-1 in two species of laboratory rodents. Only in experiments with subacute treatment of mice with ENU, the number of PCEs decreased significantly by 15.7%, which is an evidence of slight toxic effect. The cells of rats were stained with acridine orange unlike murine cells that were stained with May-Grünwald-Giemsa stains. In the frames of the US NTP it was found that non-DNA-specific stains (e.g., May-Grünwald-Giemsa) are not able to distinguish MN from basophilic mast granules present in rats bone marrow unlike DNA-specific stains (e.g. acridine orange) which leads to false-positive results (8).

The data obtained in the comet assays are presented in Table 3. The viability of various kinds of cells were assessed by the trypan blue dye exclusion technique. This parameter was 82%-85% in hepatocytes and kidney cells due to mechanical damage.

Table 3. DNA migration in peripheral blood leucocytes, kidney, and liver cells of mice after acute and subacute treatments with FS-1 (means \pm SD)

Groups of mice with various treatments (dose in ml/kg or mg/kg)	Treatment	% tail DNA in peripheral blood leucocytes	% tail DNA in liver cells	% tail DNA in kidney cells
FS-1 (10)	Acute	2.16 \pm 0.93	2.83 \pm 1.14	3.17 \pm 1.15
	Subacute	1.16 \pm 0.63	2.66 \pm 0.94	3.09 \pm 1.15
FS-1 (5)	Acute	1.58 \pm 0.96	3.33 \pm 0.99	3.87 \pm 1.14
	Subacute	1.17 \pm 0.76	3.17 \pm 0.91	3.87 \pm 1.14
FS-1 (2.5)	Acute	1.00 \pm 0.53	3.08 \pm 1.29	3.08 \pm 0.93
	Subacute	1.23 \pm 0.73	3.50 \pm 1.46	2.86 \pm 0.73
Positive control ENU (50 mg/kg)	Acute	9.67 \pm 1.02*	16.83 \pm 3.55*	18.67 \pm 3.77*
	Subacute	9.72 \pm 1.02*	16.89 \pm 3.17*	18.72 \pm 3.27*
Negative control (10)	Subacute	0.99 \pm 0.60	2.33 \pm 0.76	2.83 \pm 1.02

* $p < 0.001$ (Mann-Whitney U-test and Kruskal-Wallis test with Dunn's multiple comparison test) compared with respective negative control; the doses of treatment were ml/kg except ENU (ethylnitrosourea) – mg/kg; in all groups were 6 rodents

No exceed of migration of DNA (% DNA in tail) was found in peripheral leucocytes, kidney and liver cells of mice treated with FS-1 according to two protocols. The endpoint used in our comet experiments was percentage of DNA in tail which is most precise indicator of DNA damage (13). The results obtained in our experiments concerning negative (baseline) and positive controls (ENU) in both assays are in agreement with the results of other investigators and our historical data (10-12, 18).

DISCUSSION

The results obtained in our experiments suggest that newly synthesized compound FS-1 lacks genotoxic activity since it was applied to mice and rats at repeated doses of 40%, 20% and 10% of MTD and completely negative results were obtained in the MN test in bone marrow PCEs (rats and mice) and the comet assay in murine leucocytes, hepatocytes and kidney (renal) cells. Both tests are widely used to study genotoxic activity of natural and synthesized agents (12, 13, 17, 19).

Our findings are not unexpected since FS-1 is an iodophor. Other iodophors, e.g. povidone-iodine and Wescodyne, are broad-spectrum microbicides with activity against bacteria (including *Mycobacterium tuberculosis*), viruses (including HIV-1 or AIDS), fungi, and protozoa. They consist of elementary or ionized iodine bound to polymer carriers (such as poly-1-vinyl-2-pyrrolidone and dextrans), which increase solubility and provide a reservoir of iodine (1, 5). 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, iodophors are used as potent microbicides (1, 5). The present results are in agreement with our recent data concerning the evaluation of DNA damaging activity of the compound in human lymphocytes and 2 human tumor cell lines, HeLa and Caco-2 (7).

Since the tested compound, FS-1 is iodophor, it would be of interest to stress that widely used representative of this class 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 (20-22). These findings support the results obtained in this study. Povidone-iodine can be used as nasal spray because of low toxicity and good tolerance. FS-1 is planned to be investigated in clinical trials with HIV and HPV infected subjects (oral and topical applications, respectively).

Concerning genotoxic activity of FS-1, it should be indicated that the compound is not mutagenic in the *Salmonella*/microsome test (unpublished data), MN, and the comet assays *in vitro* in human and mouse cancer cells lines (6, 7). In addition, our *in vivo* experiments in mice and rats using MN and comet assays confirmed the absence of FS-1 genotoxicity.

Based on the results of the analyses of the literature data carried out by Brambilla et al. (8) it can be assumed that the compounds with probability of at least 63% does not possess carcinogenic properties.

These results are very important for the next level of genotoxicity studies of FS-1, which can be used in clinical medicine after additional testing of safety for humans.

Conflict of interest

We declare no conflicts of interest.

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