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ORIGINAL ARTICLE

Genotoxic activity of the new pharmaceutical FS-1 in *Salmonella*/microsome test and mouse lymphoma L5178Y cells

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Summary

Purpose: *The purpose of this study was to determine possible genotoxic effects of a new very promising antibacterial/antiviral drug FS-1.*

Methods: *The drug was tested in TA98, TA100, TA102, TA1535 and TA1537 strains of Salmonella (Ames test) with and without metabolic activation, and also in mouse lymphoma L5178Y cells by means of micronucleus and comet assays. In microbes the drug was tested at concentrations up to 500 μg/plate and in mouse lymphoma cells up to 2,000 μg/ml.*

Results: *In both test-systems in all experiments completely negative results were obtained although FS-1 was tested at maximum tolerated doses.*

Conclusions: *The drug is not genotoxic. This is advantageous because many antibacterial/antiviral drugs possess such activity.*

Key words: *antibacterial/antiviral drug, comet assay, micronucleus test, mouse lymphoma L5178Y, Salmonella/microsome assay*

Introduction

An effective drug against a number of strains of microbes and viruses was recently synthesized and patented (KZ Patent No. 15116) in Kazakhstan. This drug called FS-1 is a complex of iodine with synthesized polysac¬charides, i.e., it is an iodophor, a combination of iodine and a solubilizing agent that releases free iodine when diluted with water. Iodophors pos¬sess a quick microbiocide action against a wide variety of microorganisms such as bacteria, viruses, fungi, and protozoa [1]. Recently it was shown that the compound is very active against HIV-1 virus *in vitro* inhibiting its replication [2]. Very interesting and promising results were obtained from experiments with Ehrlich ascites carcinoma in mice [3]. FS-1 alone at dose of 10 ml/kg prolongs the lifespan of tumor-bearing mice by 15% while doxorubicin alone prolonged it by 188%. Combination of the two drugs leads to synergistic therapeutic effect – life span of tumor-bearing mice increased by 286% [3].

At present FS-1 is under clinical trials in Kazakhstan.

Because of its low toxicity in rodents [i.e., 25 ml/kg is the maximum tolerated dose (MTD) for both mice and rats] it is possible to use FS-1 in clinical medicine, if it meets all safety criteria. These criteria include, in the first turn, absence of genotoxic activity of the potential pharmaceutical [4].

FS-1 was studied for its ability to induce DNA damage (by means of the comet assay) and micronuclei (MN) in human tumor cell lines HeLa and Caco-2 at concentrations of 200, 500, and 1000 μg/ml without exogenous metabolic activation [5]. The compound was additionally tested for

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DNA damaging ability (the comet assay) in human lymphocytes at concentrations of 200, 400 and 800 μg/ml also without metabolic activation. Neither DNA damage nor MN formation was observed after treatment of all types of cells with FS-1 [5].

Furthermore, DNA-damaging and MN inducing activity of FS-1 was studied in *in vivo* systems in rats and mice. DNA-damaging activity of FS-1 was investigated in leucocytes, hepatocytes and kidney cells of mice. MN induction of the compound was studied in bone marrow cells of rats and mice [6]. No activity was found in any test system.

In the US FDA Guidelines for testing pharmaceuticals it is indicated that all potential drugs ought to be tested with bacterial mutagenicity assay (Ames assay with *Salmonella*) with and without metabolic activation, in mammalian cells with *in vitro* MN and the comet assays with and without metabolic activation (preferably in mouse lymphoma L5178Y cells), and in *in vivo* MN assay in bone marrow of mice [4].

For complete battery of genotoxicity studies, FS-1 should be studied in the Ames assay and in mouse lymphoma L5178Y cells.

The aim of the present study was to assess the activity of FS-1 to induce reverse mutation in *Salmonella*/microsome assay, and also MN formation as well as DNA-damaging effects by means of the comet assay in mouse lymphoma L5178Y cells.

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 and Taufkirchen, Germany). 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 similar composition as iodine-lithium-alpha-dextrin used in some countries as a potent antimicrobial/antiviral agent [7].

Ames test

The incorporation version of the Ames test was performed according to the recommendations of Maron and Ames [8], following the principles of OECD guideline 471 [9]. Five *Salmonella* typhimurium histidine-auxotrophic strains TA98, TA100, TA102, TA1535 and TA1537 were used for the assay. Cultures of each test strain were prepared from their main strain plates and then used in the late exponential growth phase. Each primary experiment included one negative control (at the same time solvent control, distilled water, 100 μl/plate), one positive control for each strain, and four concentrations of FS-1 (500, 125, 62.5 and 31.3 μg/plate). The highest dose (500 μg/plate) was the maximal tolerated dose for all strains of *Salmonella*. The test substance was assessed in three independent experiments, each conducted in the absence and presence of the S9 metabolic activation system from rat livers (Aroclor 1254, Sigma-Aldrich), using triplicate plates for all test substance concentrations. Briefly, 0.1 mL test substance, 0.1 mL bacterial culture and 0.5 mL of S9 mix were added to 2 mL molten agar at 42° C and poured onto Vogel Bonner-E minimal glucose agar plates. The plates were inverted and incubated for 65–70 hrs at 37° C in the dark. Revertant colonies were counted, and the background lawn was inspected for signs of either toxicity or compound precipitation. Positive controls for *Salmonella* without the S9 fraction were 2-nitrofluorene (2-NF, 0.1 μg/plate) for TA98, TA100 and TA1535, cumene hydroperoxide (100 μg/plate) for TA102, sodium azide (NaN_3, 1 μg/plate) for TA1537 [10,11]. In presence of S9 for all strains, 2-aminoanthracene 2 μg/plate was used [10,11].

Mouse lymphoma L5178Y cells

L5178Y cells were handled as previously described [12]. Briefly, cells were cultured in suspension in RPMI 1640 medium supplemented with antibiotics (95 units/ml penicillin, 95 μg/ml streptomycin), 0.25 mg/ml glutamine, 107 μg/ml sodium pyruvate and 10% heat-inactivated horse serum (all chemicals and reagents used in all experiments were from Sigma-Aldrich, Taufkirchen, Germany). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37 °C. To study the DNA-damaging activity first the toxicity of the compound must be evaluated because the tested substances should be used at doses which induce death in about 50-60% of cells (otherwise in case of non-toxic compound which is the case it should be tested at doses about 2000 μg/ml compared to the negative control). The experiments were conducted in flasks containing 5 ml of RPMI with $2x10^6$ lymphoma cells and incubation time 18 hrs at concentrations of 2000, 1000 and 500 μg/ml. The cells' number was evaluated by means of cells counter (Coulter, UK). Since we could not obtain signs of substantial toxicity, the compound was used in the mentioned concentrations (the number of live lymphoma cells was between 87.4 and 68.9% of the negative control – normal saline). In all experiments FS-1 was tested at concentrations 500, 1000 and 2000 μg/ml.

Study of MN-inducing activity of compounds in mononucleated L5178Y cells

Exponentially growing mouse lymphoma L5178Y cells ($1x10^6$ in 5.0 ml of medium in 25.0 ml flasks) were treated for 18 hrs with the test substance. After incuba-

tion the cells were washed with PBS buffer, collected by centrifugation (5 min at 1000 rpm), and placed on glass slides by cytospin centrifugation. Fixation was performed with methanol at –20 °C for 1 h. For staining, the slides were washed with PBS buffer, incubated for 3 min with a solution of 62.5 μg/ml acridine orange in Soerensen buffer (67 mM Na_2HPO_4/KH_2PO_4, pH 6.8), and washed again with PBS buffer. The numbers of nuclei and MN were scored using fluorescence microscopy. For each concentration of the compound two parallel cultures of the cells were prepared. Two slides were prepared from each culture. One thousand cells per slide were evaluated. As a positive control mitomycin C (MMC) was used at concentration 0.0625 μg/ml [12]. Cells with MN and the total number of MN were calculated.

Study of MN-inducing activity of compounds in binucleated L5178Y cells

Cells were incubated with the compounds and cytochalasin B (6 μg/ml, 20 hrs). Cytochalasin B was added to cell suspensions immediately after the compounds as was previously described [12]. The rest of the procedures was the same as it was described in our previous publication [12]. Except registration of MN and cells with MN, apoptotic and necrotic cells as well as cells with nucleoplasmic bridges and nuclear buds were evaluated as recommended for MN cytome assay in lymphocytes [13]. In addition, cytokinesis-block proliferation index (CBPI) was calculated according to guidelines [1]. MMC was used as a positive control at concentration of 0.0625 μg/ml.

Study of MN-inducing activity of compounds in binucleated L5178Y cells after metabolic activation

The toxic effect of the compound was assessed by counting the living cells and the total number of cells in suspension by means of cell counter (Vi-CELL, Beckman Coulter, UK).

It is known that many chemical agents need metabolic activation for their biological activity (toxicity, mutagenicity, carcinogenicity). We used artificial metabolic activation system (S-9 mix) widely used in biological experiments to assess possible metabolites of the compounds according to the published protocol [14]. The final concentration of the S-9 mix (activation mixture) in the medium was 2%. The composition of the S-9 mix was as follows: 108 mg of glucoso-6-phosphate, 15 mg of NADP, 0.6 ml of 150 mM KCl, 1.2 ml of bi-distilled water, 1.2 ml of S9 fraction. As positive control cyclophosphamide was used at concentration of 5.0 μg/ml, because it needs metabolic activation to produce biological effects. FS-1 was incubated with mouse lymphoma L5178Y cells (1x106 cells in 5.0 ml of medium) at concentrations of 1000 and 2000 μg/ml, then washed, placed in new medium, and cytochalasin B was added (6 μg/ml). Incubation lasted 20 hrs.

Study of DNA-damaging activity with the Comet assay

This assay was carried out as described in our publication [12]. Briefly, fully frosted microscope slides, coated with a layer of high melting-point agarose (1.5%, diluted in calcium- and magnesium-free phosphate-buffered saline), were used. Twenty microliters of the cell suspension were diluted in 180 μl of low melting-point agarose (0.5% diluted in calcium- and magnesium-free phosphate-buffered saline), and 45 μl of the suspension were embedded on the frosted microscope slides. Slides were then immersed in a jar, containing fresh cold lysing solution (1% Triton X-100, 10% DMSO, 89%: 10 mmol/l Tris, 1% Na sarcosine, 2.5 mol/l NaCl, 100 mmol/l Na2EDTA, pH 10.0) for lysis, at 4 °C in a dark chamber for 1 h. Next, slides were placed into a horizontal electrophoresis tank with fresh alkaline electrophoresis buffer (300 mmol/l NaOH, 1 mmol/l Na2EDTA; pH 13) and left for 20 min at 4 °C in the dark, to allow DNA unwinding and alkali-labile damage expression. Electrophoresis was then carried out at 4 °C in the dark, for 20 min in a 25 V and 300 mA electrical field. Afterwards the slides were neutralized for 5 min in 0.4 mol/l Tris (pH 7.5) and the DNA was stained by adding 20 μl of 20 μg/l propidium iodide. FS-1 was tested at three concentrations, namely 2000, 1000 and 500 μg/ml after 20 hrs incubation, and the experiments were carried out two times. As a positive control methyl metansulfonate (MMS) was used (30.0 μg/ml, only 3 hrs treatment because of high potency of this agent to induce genotoxic effect).

A fluorescence microscope at 200x magnification and a computer-aided image-analysis system (Komet 5, Kinetic Imaging Ltd., Liverpool, UK) were used for analysis. Fifty cells in total (25 per slide) were analyzed and the results were expressed as %DNA in the tail. The experiment was repeated twice.

Statistics

The mutagenicity experiments (Ames test) were performed three times in at least triplicate per concentration. The data are presented as the arithmetic mean percentage ± standard deviation (SD) compared to the control group, which is the unexposed bacterial strain. Statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using Graph-Pad InStat software (GraphPadSoftware Inc., La Jolla, USA). The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlett´s test, respectively. The differences were considered significant at $p < 0.05$.

Statistical analysis of results obtained in the comet assay was done by the software of a computer-aided image-analysis system (Komet 5, Kinetic Imaging Ltd., Liverpool, UK).

Online chi-square test with Yate's correction was applied to compare the results of the experiments on

MN-inducing activity of FS-1 with positive and negative controls. (http://www.graphpad.com/quickcalcs/contingency1.cfm)

Results

The results of the experiments with five strains of *Salmonella* are presented in Tables 1 and 2. It can be noticed that FS-1 at doses between 31.3 and 500 μg/plate did not induce mutagenic effect (gene reverse mutations) in all five strains of *Salmonella*.

The highest concentration of FS-1 which was slightly toxic for lymphoma cells was 2000 μg/ml (the number of living cells was 75% in experiments without metabolic activation and 88% with metabolic activation). The decrease of live cells' number after 20 hrs incubation with the compound was between 12 and 20%, which is acceptable for experiments with MN induction and DNA damage in the comet assay.

FS-1 did not induce cells with MN and it also increased of total number of MN in mononuclear lymphoma cells even at the highest concentration (2000 μg/ml, Table 3). MMC used as a positive control increased the number of cells with MN and the total number of MN by 3.9-fold, which is comparable with the results published by another research group [15].

In binucleated lymphoma cells the compound also did not induce an increase in the number of cells with MN as well as the total number of MN in all three experiments without metabolic activation (Table 4). As can be seen, spontaneous levels of cells with MN in experiments without cytochalasin B were 2-3-fold less than in experiments with it.

In our experiments we also studied other parameters reflecting genotoxic events, such as frequencies of buds (which reflect amplification of DNA) and bridges (which reflect appearance of dicentric chromosomes) [13]. Cytokinesis-block proliferation index (CBPI) did not differ significantly between the experimental groups. No increase of any of the mentioned cytome parameters was observed. MMC used as a positive control (recommended by OECD Guidelines 487 [16]) induced between 2.4- and 2.8-fold increase of the numbers of cells with MN, total number of MN and nuclear buds. As for nuclear bridges, they were significantly elevated only in the positive control group (3.5‰) while this parameter was equal to 0 in all other groups (Table 4).

The same negative results were obtained in the experiments with metabolic activation of the compound (Table 5). In this case cyclophospha-

Table 1. Results of the Ames test conducted with FS-1 (mean of three independent experiments)

Treatment and doses	*Strains of Salmonella*			
	TA98		*TA100*	
	-S9	*+S9*	*-S9*	*+S9*
FS-1 (500 μg/plate)	15 ± 5	38 ± 10	122 ± 16	118 ± 12
FS-1 (250 μg/plate)	19 ± 4	31 ± 7	132 ± 11	144 ± 16
FS-1 (125 μg/plate)	14 ± 2	29 ± 4	119 ± 15	132 ± 10
FS-1 (62.5 μg/plate)	20 ± 5	28 ± 4	135 ± 11	143 ± 10
Positive control	1055 ± 211[a]	1245 ± 158[b]	1323 ± 96[a]	1128 ± 176[b]
Solvent control	18 ± 6	31 ± 7	108 ± 11	119 ± 17

[a] 2-nitrofluorene (0.1 μg/plate), [b] 2-aminoanthracene (2 μg/plate), solvent control 100 μl of bidistilled water

Table 2. Results of the Ames test conducted with FS-1 (mean of three independent experiments)

Treatment and doses	*Strains of Salmonella*					
	TA102		*TA1535*		*TA1537*	
	-S9	*+S9*	*-S9*	*+S9*	*-S9*	*+S9*
FS-1 (500 μg/plate)	244 ± 44	287 ± 77	11 ± 5	13 ± 3	11 ± 2	14 ± 3
FS-1 (125 μg/plate)	223 ± 31	301 ± 65	15 ± 3	16 ± 4	8 ± 2	12 ± 3
FS-1 (62.5 μg/plate)	274 ± 48	249 ± 59	16 ± 3	11 ± 2	14 ± 4	15 ± 3
FS-1 (31.25 μg/plate)	218 ± 32	285 ± 47	13 ± 5	14 ± 5	11 ± 2	18 ± 6
Positive control	631 ± 33[a]	588 ± 29[b]	304 ± 51[c]	115 ± 12[b]	215 ± 34[d]	286 ± 21[b]
Solvent control	247 ± 22	276 ± 43	12 ± 4	15 ± 3	118 ± 12	10.3 ± 2

[a] Cumene hydroperoxide (100 μg/plate), [b] 2-aminoanthracene (2 μg/plate), [c] 2-nitrofluorene (0.1 μg/plate), [d] sodium azide 1 μg/plate, solvent control 100 μl of bidistilled water

Table 3. Micronucleus inducing activity of FS-1 in mononuclear mouse lymphoma L5178Y cells (mean of 3 experiments, treatment 20 hrs)

Agent	*Concentration (μg/ml)*	*Cells with MN (‰)*	*Total number of MN per 1000 cells*
FS-1	500	5.6 ± 1.5	5.6 ± 1.5
	1000	7.3 ± 1.8	7.3 ± 1.8
	2000	6.6 ± 2.4	8.3 ± 2.8
Positive control (MMC)	0.0625	23.3 ± 1.8*	28.3 ± 1.9*
Negative control (PBS)	-	6.0 ± 1.2	6.0 ± 1.2

*p<0.001; (x^2 test with Yates's correction), MN: micronuclei, MMC: mitomycin C, PBS: phosphate buffered saline

mide was used as a positive control since it needs metabolic activation to induce biological effects and is indicated as a recommended control in *in vitro* MN studies in mammalian cells [16]. As in previous experiments, FS-1 did not show any evidence of activity. In the positive control group the rates of cells with MN and the total number of MN were 3.4- and 4-3-fold higher than in the negative control group. The rate of nucleoplasmic bridges reflecting dicentric chromosomes (chromosomal rearrangement) was 6.5‰ and 0 in all other cases.

The results of the comet assay are presented in Table 6. As it can be seen, in these experiments also FS-1 was not active at any concentration unlike the positive control–MMS.

Discussion

In our experimental study FS-1 was tested in a bacterial test system which is able to register gene mutations and mammalian cells (mouse lymphoma L5178Y) by means of MN assay with and without metabolic activation system and the comet assay. In all test-systems applied in the present study absolutely negative results were obtained. It is also noteworthy, that FS-1 does not induce MN in bone marrow cells of rats and mice [6] and also MN and DNA damage in human cancer cell lines Caco-2 and HeLa [5].

It is noteworthy that the frequencies of spontaneous gene mutations in all bacterial strains as well as those induced by positive controls were within the ranges described by other investigators [8,10,11].

The absence of genotoxic activity of the potential drug is not surprising. The main components of the compound, iodine and polysaccharides are not genotoxic. Recently we reviewed the literature data concerning possible genotoxicity and carcinogenicity of iodine and its derivatives and did not find any evidence of genotoxic activity of these compounds in the literature [17]. Also all investigated polysaccharides are non-genotoxic and non-carcinogenic [18]. They even possess an-

Table 4. Micronuclei, other nuclear anomalies and cytokinesis blocked proliferation index of FS-1 in binuclear mouse lymphoma L5178Y cells (mean of 3 experiments, treatment 20 hrs)

Treatment (μg/ml)	*Cells with MN (‰)*	*Total number of MN*	*Buds*	*Bridges*	*CBPI*
FS-1 (500)	22.5 ± 1.7	22.5 ± 2.1	0	0	2.03 ± 0.9
FS-1 (1000)	24.0 ± 1.2	24.3 ± 5.4	0.5 ± 0.3	0	2.00 ± 1.2
FS-1 (2000)	18.3 ± 1.6	19.8 ± 3.5	0.5 ± 0.3	0	1.96 ± 1.5
Positive control MMC (0.0625)	50.5 ±5.6*	61.5 ± 1.9*	5.5 ± 2.2*	3.5 ± 1.2*	1.88 ± 2.0
Negative control (PBS)	20.9 ± 2.6	22.3 ± 1.9	2.0 ± 1.2	0	1.06 ± 2.1

*p<0.001 (x^2 test with Yates's correction), PBS: phosphate buffered saline, CBPI: cytokinesis-block proliferation index, MN: micronuclei, MMC: mitomycin C

Table 5. Micronuclei, other nuclear anomalies and cytokinesis blocked proliferation index of FS-1 in binuclear mouse lymphoma L5178Y cells after metabolic activation (treatment for 3 hrs with S-9 mix, then cytochalasin B treatment for 20 hrs, mean of 3 experiments)

Treatment (μg/ml)	*Cells with MN (‰)*	*Total number of MN*	*Buds*	*Bridges*	*CBPI*
FS-1 (1000)	15.5 ± 1.6	16.3 ± 1.5	8.5 ± 2.0	0	2.03 ± 1.3
FS-1 (2000)	17.6 ± 2.2	18.5 ± 1.9	6.3 ±1.6	0	1.95 ± 1.8
CP (5.0)	44.8 ± 5.8*	55.8 ± 9.6*	4.5 ± 1.4	6.5 ± 1.9*	2.05 ± 2.1
Negative control (PBS)	13.0 ± 1.6	13.0 ± 1.6	8.3± 2.2	0	2.16 ± 1.7

*p<0.001 (x^2 test with Yates's correction), PBS: phosphate buffered saline, CBPI: cytokinesis-block proliferation index, CP: cyclophosphamide

Table 6. DNA-damaging activity of FS-1 in mouse lymphoma L5178Y cells
(Comet assay)

Treatment (concentration, μg/ml)	*DNA in tail, %*
Experiment 1	
FS-1 (500)	4.2 ± 0.5
FS-1 (1000)	4.8 ± 0.4
FS-1 (2000)	5.7 ± 0.4
MMS (30)	42.4 ± 3.1*
PBS	4.6 ± 0.5
Experiment 2	
FS-1 (500)	3.8 ± 0.7
FS-1 (1000)	4.1 ± 0.3
FS-1 (2000)	4.0 ± 1.3
MMS (30)	33.6 ± 2.9*
PBS	2.7 ± 0.3

*p<0.001. MMS: methyl methanesulfonate, PBS: phosphate buffered saline

tioxidant and antimutagenic properties and they potentially can be used as anticancer agents [18].

Since FS-1 is iodophor, it would be of interest to mention that povidone-iodine, widely used representative of this class of preparations, is non-genotoxic in several *in vitro* mutagenicity tests in bacteria [19] and mammalian cells [20] as well in mouse bone marrow MN assay [21]. These findings support the results obtained in this study.

Genotoxicty tests are useful tools in the investigation of possible harmful effects of drugs (i.e. carcinogenicity). Brambilla et al. [22] reviewed the literature data concerning DNA-damaging activity of 146 pharmaceuti¬cals widely used in modern medicine and showed 63.1% concordance in the case of DNA strand breaks and carcinogenicity of the drugs. The same authors reviewed the literature data on genotoxicity of 48 antibacterial, antiviral, antimalarial and antifungal drugs [23]. They found that concordance between carcinogenicity and positive genotoxic effects for these widely used in clinical practice drugs in *in vitro* and *in vivo* cytogenetic tests, bacterial mutagenicity tests and DNA damaging activity was 70%, 50%, 50% and 43%, respectively.

In fact, FS-1 was tested in all test-systems which are recommended by the US FDA for testing of pharmaceuticals (Ames assay, MN assay in mammalian cells *in vivo* and *in vitro*, DNA-damaging activity in mammalian cells) with absolutely negative results [4]. It means that FS-1 is safe from the genotoxicity point of view. This statement is also supported by the very low toxicity of FS-1 (25 ml/kg for rats and mice) and also the data of literature [22,23].

It is noteworthy that FS-1 will be applied in clinical trial as anti-HIV-1 agent. Almost all drugs used nowadays in the treatment of HIV-1 are genotoxic and some of them are carcinogenic for rodents [24]. In case of efficacy of FS-1 in treatment it will have an advantage as a non-genotoxic drug.

A recently described synergistic effect of FS-1 [3] and some cytostatic drugs against murine cancer warrant further investigations for potential application in cancer chemotherapy.

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