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CYTOTOXICITY AND ACUTE TOXICITY OF A NEW COMPOUND COMPRISING IODINE ADDUCTS IN MICE

Abstract. The cytotoxicity and acute toxicity were studied after oral and intraperitoneal administration of a new compound (R8) comprising iodine adducts to mice. The median cytotoxic concentration (CC_{50}) was determined in the six cell lines, and ranged from 2.1 to 3.9 mg/ml. The median lethal dose of the compound R8 in mice when administered orally was 1000 mg/kg, intraperitoneally – 200 mg/kg. According to the international system of classification for toxicity of chemicals GHS, the compound R8 may be assigned to Toxicity Category IV.

Keywords: iodine, adducts, cytotoxicity, acute toxicity, mice.

Introduction. The prevalence of resistance to colistin and carbapenems in bacteria actually disarms the modern anti-infectious therapy [1]. For this reason, the urgent is the search for new approaches and strategies for the treatment of drug-resistant infections [2]. Currently, iodine is among the few substances that have retained antimicrobial properties [3-6]. The preparations comprising complexes of iodine with organic macromolecules such as povidone-iodine and cadexomers, have become widespread in human and veterinary medicine [5, 7]. In addition, the complexes of iodine with dextran increase expression of IL-1β, IL-8, TNF-α, and VEGF mRNA and do not affect IL-6, IL-10, IL-12 and bFGF mRNA, which indicates the specific immunotropic activity [8]. At the same time, their use is limited to relatively high chemical activity and toxicity of povidone-iodine [7,9]. One of the ways to reduce iodine toxicity lies in its combining with carbohydrates and producing a complex with dextrin (cadexomers). A comparative study on the effect of various complex compounds of iodine on the blood vessels in the chick embryo chorioallantoic membrane showed that povidone-iodine at 1% concentration is characterized by strong irritating activity, whereas cadexomer at a concentration up to 1.8% had a moderate activity with an exposure for 60 minutes [9]. This approach was used in the synthesis of a new stable iodine-containing substance. A new coordination iodine compound (R8) has been synthesized in the Scientific Center for Anti-Infectious Drugs [10]. To carry out initial assessment of toxicity of the compound R8, the cytotoxicity was studied in various mammalian cell cultures, and acute toxicity studies were performed in mice.

Materials and Methods. *Test substance*. Coordination iodine compound with molecular weight of 458.11 and the chemical formula $C_{18}H_{23}I_3N_2O_4$. The substance at 20°C and 101.3 kPa is a solid crystalline gray-green powder with a faint iodine odor. It is readily soluble in water. Immediately before performing assays, the R8 substance was dissolved in sterile water.

Cell cultures. RD - human embryonic rhabdomyosarcoma cells, BHK-21 - newborn Syrian hamster kidney fibroblasts, MDCK - dog kidney cell line, Hep G2 - human hepatocellular carcinoma cells (ATCC). The subinoculated suspension cell cultures: H9 – human cutaneous T-cell lymphoma (ATCC), K562 - human erythroblastoid leukemic cells (ATCC).

Laboratory animals. White female outbred mice in the number of 24 individuals, aged 1.5-2.5 months and weighing 22 g ± 10%, were obtained from the Scientific and Practical Center for Sanitary-Epidemiological Expertise and Monitoring (Almaty, Kazakhstan). The mice were kept in individually ventilated cages (IVC) (Tecniplast, Italy) in controlled conditions (with a 12- hour day/night cycle, at a temperature of 22 ± 2°C and relative humidity of 55 ± 10%). The mice were fed the commercial complete feed ("Assortiment-Agro" LLC, Russia). After a 2-week acclimatization period, the animals were randomized into groups. The animals were given *ad libitum* access to food and water.

Cytotoxicity. Cytotoxicity was evaluated by analyzing the cell's ability of absorbing neutral red in accordance with the OECD Series on Testing and Assessment, No. 129 "Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests". The cells were seeded in 96-well plates at a concentration of 2.5×10^5 cells in 1.0 ml. The plates were incubated in a 5% CO_2 incubator at 37°C. After incubation for 24 hours, the culture medium was removed from the wells, and 200.0 µl of the incomplete medium containing the test substance R8 were added to each well. The R8 concentrations were prepared by double dilution with DMEM medium (for HepG2, RD, 21-BHK, MDCK cell cultures) or with RPMI-1640 medium (for H9, R562 cell cultures), starting from 10.0 mg/ml. 200.0 µl of incomplete culture medium (RPMI-1640 for H9, K562 and DMEM for HepG2, RD, 21-BHK, MDCK) were added to each negative control well. For HepG2, RD, BHK-21 or MDCK cell cultures, the culture medium with the substance was removed from the wells after 48 and 72 hours, and 150.0 µl of Dulbecco's Phosphate Buffered Saline (DPBS) were added to each well (to wash the cells). After completion of the washing step, the supernatant was removed from the plate, and 100.0 µl of the neutral red working solution were added to each well. The plate was incubated for 3 hours at 37°C. After incubation of the plate, the supernatant was removed and 150.0 µl of DPBS was added (to wash the cells). Further, the supernatant was removed from the plate, and 150.0 µl of lysis solution (mixture of 1% glacial acetic acid, 50% ethanol and 49% distilled water) were added to each well. For K562 or H9 cell cultures, the plate was centrifuged after 48 and 72 hours at 130 g for 5 minutes; the culture medium with the substance was further removed from the wells of the plate, and 250.0 µl of the neutral red working solution were added to each well. The plate was incubated for 3 hours at 37°C. After incubation, the plate was centrifuged at 130 g for 5 minutes; the supernatant was further removed from the wells of the plate, and 250.0 µl of the washing/fixing solution (0.5% formaldehyde, 99.5% distilled water) were added to each well. The plate was centrifuged at 130 g for 2 min, the supernatant was removed, and 100.0 µl of lysis solution were added. The optical density in the wells was measured with a microplate reader Tecan Sunrise RC.4 (Austria) at a wavelength of the main filter of 540 nm. Evaluation of the results and calculation of the median cytotoxic concentration (CC_{50}) were carried out based on the methods described in [11, 12].

Acute toxicity. Acute toxicity was evaluated according to the OECD Guidelines for the Testing of Chemicals, Test No. 423: Acute oral toxicity - Acute toxic class method. The test substance R8 was administered intragastrically and intraperitoneally at doses of 50, 300, and 2000 mg/kg. Toxicity category was assigned for the compound R8 as indicated in the international classification system for toxicity of chemicals GHS, according to Table 1.

The body weight of animals was measured weekly, starting on the first day of the study and until euthanasia following the completion of the experiment. The clinical signs of poisoning were recorded. The mice were killed by cervical dislocation. At necropsy, a macroscopic examination of organs was performed. The experiments on animals were approved by the Ethics Committee of the Scientific Center (No. 04-03 037) dated 26.1.2016, and Local Ethics Committee of the S.D. Asfendiyarov KazNMU dated 24.02.2016 (Protocol No. 2).

Table 1 – GHS classification of toxic chemicals

Toxicity category	Category 1	Category 2	Category 3	Category 4	Category 5
LD_{50}, mg/kg	< 5	> 5 < 50	> 50 < 300	> 300 < 2000	> 2000 < 5000

Statistical data processing. The means and standard deviations were calculated for the body weight of mice. The Mann-Whitney test for significance level at $p < 0.05$ was used to test the null hypothesis.

Results and Discussion. *Cytotoxicity.* The results of cytotoxicity evaluations for R8 on various cell cultures after 48 and 72 hours of incubation are shown in Table 2.

Table 2 – Results of determining cytotoxic effects of R8 in the neutral red test

Cell culture	CC_{50}, mg/ml	
	48 h	72 h
MDCK	3.8	3.9
HepG2	3.7	3.6
RD	2.4	2.1
BHK-21	2.1	2.1
H9	3.1	2.3
K562	2.2	1.05

Despite rather wide range of variability in the results of determining CC_{50} for R8 within 2.1 to 3.8 mg/ml after 48 hours of incubation, these concentrations are close to 5 mg/mL (or 10 mM), which indicates the relative nontoxicity of the compound [13]. For comparison, the concentration of povidone-iodine at which 50% of murine fibroblasts (L929) survive after a 30-min incubation in the neutral red assay is 460 - 490 mkg/ml or 1.8 - 1.9 mM of iodine [14]. Thereby, the new iodine adduct exhibits lower cytotoxicity as compared with povidone-iodine, even with long-lasting incubation. To evaluate the R8 safety, the acute toxicity was examined using two routes of administration, enteral and parenteral.

Acute toxicity after the intragastric administration. After administration of an initial dose of R8 (300 mg/kg), none of the animals died within 14 days. In the absence of a lethal effect, the administered dose was increased to 2000 mg/kg. After the intragastric administration of the R8 substance at a dose of 2000 mg/kg, one mouse died after 24 hours and one more mouse died 48 hours later. In total, 2 mice died in the experiment. The data obtained are shown in Table 3.

Table 3 – Mortality of mice after the intragastric administration of R8

Groups	Dose, mg/kg	Effect (died/initial number)	Time of death
Control	–	0/3	–
Experimental 1	300	0/6	–
Experimental 2	2000	2/3	Days 1 and 2

To evaluate the adverse effect of R8 in mice from the group with the dose of 300 mg/kg, the dynamics of body weight was studied in the experiment (Table 4).

Table 4 – Dynamics of body weight in mice after the intragastric administration of R8

Groups	Dose	Average weight of animals, g	
		Day 1	Day 14
Control	Solvent (water)	19.80±0.35	24.7±1.8
Experimental 1	300 mg/kg	21.4±0.75	25.2±1.1

The positive dynamics of body weight and absence of clinical signs to confirm toxic effect after exposure to 300 mg/kg suggest that LD_{50} for R8 is about 1000 mg/kg.

On day 14 the animals were killed by cervical dislocation and subject to necropsy. The macroscopic examination of internal organs of experimental animals did not reveal any pathological deviations. At the same time, the arrangement of internal organs was normal; no cohesion between them or sharp increase or decrease in size were observed. The liver was deep red, with smooth capsule, homogeneous on the cut surface, the consistency was normal. Hemorrhages or fat deposits in the liver parenchyma were not detected. The loops of the small intestine were free. The walls of the intestine and bladder were not damaged.

The renal capsule was easily removed. The spleen was deep cherry in color, does not produce scrape-off on the cut surface, non-ulcerated and without signs of hemorrhages. The heart muscles were dark red, homogeneous, free of injuries. Hemorrhages were not detected. The lung tissue was airy, pink, with no signs of edema, hemorrhages or necrosis on the cut surface.

Acute toxicity after the intraperitoneal administration. A single-dose intraperitoneal administration of R8 at a dose of 300 mg/kg on day 3 resulted in the death of one mouse (Table 5). Immediately after the administration of R8, the animals bunched up. Within two days before the death, the mice developed arched back and exhibited a significant reduction in motor activity. Thereafter, the dose of R8 was reduced to 50 mg/kg. After administration of R8 at a dose of 50 mg/kg the mice bunched up within two hours. These symptoms gradually disappeared on the third hour. Mortality was not observed (Table 5).

Table 5 – Mortality of mice after the intraperitoneal administration of R8

Groups	Dose, mg/kg	Effect (died/initial number)	Time of death
Control	–	0/3	–
Experimental 1	300	1/3	Day 3
Experimental 2	50	0/6	–

The positive dynamics of body weight (Table 6) and the observed clinical signs of toxicity allow us to ascertain that the LD_{50} for R8 after intraperitoneal administration is about 200 mg/kg.

Table 6 – Dynamics of body weight in mice after the intraperitoneal administration of R8

Groups	Dose	Average weight of animals, g	
		Day 1	Day 14
Control	Solvent (water)	23.0±0.4	27.2±1.0
Experimental 1	50 mg/kg	23.5±0.4	25.4±1.6

All surviving mice were killed by cervical dislocation on day 14 and subject to necropsy. The macroscopic examination of internal organs of experimental animals did not reveal any pathological deviations. When examining site for injecting R8 in the right lower third of the stomach, the inflammation and commissural processes were not found. The arrangement of internal organs was normal; no cohesion between them or sharp increase or decrease in size were observed.

Correlation between the cytotoxicity and acute toxicity data. Currently, various methodological approaches were developed for using cytotoxicity test in predicting the experiments on laboratory animals. The results can be applied to the oral acute toxicity studies using oral administration, for example, to calculate the initial dose [15]. Using the regression equation for IC_{50} – LD_{50} [15] from the data on cytotoxicity for R8 (2.1 mg/ml), the expected dose was evaluated in an *in vivo* experiment. As a result, the estimated LD_{50} for R8 is 1819 mg/kg, which is higher than the experimental dose of 1000 mg/kg. These differences may be due to the mechanisms of toxic action of the compound R8.

Conclusion. The median lethal dose of the new iodine adduct R8 in mice when administered orally is 1000 mg/kg, intraperitoneally - 200 mg/kg. At that, the examined iodine adduct was less toxic as compared with iodophor - povidone-iodine. According to the international classification system for toxicity of chemicals GHS, the compound R8 may be assigned to Toxicity Category IV.

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ҚҰРАМЫНДА ИОД АДДУКТЫ БАР ЖАҢА ҚОСЫЛЫСТЫҢ ТЫШҚАНДАРҒА ЦИТОУЫТТЫЛЫҒЫ МЕН ӨТКІР УЫТТЫЛЫҒЫ

Аннотация. Құрамында иод аддуктысы бар жаңа қосылыстың (R8) тышқандарға ауызынан және перитонеальдық тәсілмен енгізгенде цитоуттылығы мен өткір уыттылығы зерттелінген. Орташа цитоуыттылық концентрациясы жасушалардың алты түрінде анықталды және 2,1 ден 3,9 мг/мл ауытқу аралығында болды. Тышқандардың орташа өлім дозасы R8 ауызынан енгізгенде 1000 мг/кг, ал перитонеальдық тәсілмен енгізгенде 200 мг/кг құрады. GHS халықаралық дәрілік заттардың уыттылық классификациясы жүйесіне сәйкес R8 қосылысын уыттылықтың 4 класына жатқызуға болады.

Түйін сөздер: иод, аддукт, цитоуыттылық, өткір уыттылық, тышқандар.

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ЦИТОТОКСИЧНОСТЬ И ОСТРАЯ ТОКСИЧНОСТЬ НА МЫШАХ НОВОГО СОЕДИНЕНИЯ СОДЕРЖАЩЕГО АДДУКТЫ ИОДА

Аннотация. Изучена цитотоксичность и острая токсичность при оральном и внутрибрюшинном способах введения мышам нового соединения (R8) содержащего аддукты иода. Средняя цитотоксическая концентрация (CC_{50}) определена на шести линиях клеток и находилась в пределах от 2,1 до 3,9 мг/мл. Средняя смертельная доза на мышах при оральном поступлении R8 составила 1000 мг/кг, при внутрибрюшинном 200 мг/кг. Согласно международной системе классификации токсичности веществ GHS соединение R8 можно отнести 4 классу токсичности.

Ключевые слова: иод, аддукты, цитотоксичность, острая токсичность, мыши.

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