World Academy of Science, Engineering and Technology
International Journal of Pharmacological and Pharmaceutical Sciences
Vol:7, No:8, 2013

Pre-Clinical Studying of Antitumor Ramon Preparation: Acute Toxicity

Raissa A. Muzychkina, Irina M. Korulkina, and Dmitriy Yu. Korulkin

***Abstract*—**In article the data of acute toxicity for pre-clinical researches of Ramon preparation is described. Ramon effects to clinical characteristics of blood, cardio-vascular system, hepatotoxic and diuretic effects were studied.

***Keywords*—**Cancer, toxicity, antitumor activity, pre-clinical testing, anthraquinones, phytopreparation, Ramon.

I. Introduction

CANCER remains as a life-threatening disease and a leading cause of death as its control has been difficult. Although, a range of conventional therapies based on chemotherapy, surgery, and radiotherapy are available, these approaches are in many cases of limited efficacy. Moreover, current anticancer regimens are frequently associated with significant levels of toxicity and the emergence of drug resistance. One major challenge to relieve cancer burden is to develop highly effective drugs with specificity on cancers but little or no side effects on normal mammalian cells.

Many research projects have been focused on developing new chemotherapies either by exploring the anticancer ability of novel compounds or by assessing drugs conventionally used in other clinical diseases [1].

Natural products have been found to be a relevant source of novel and potent bioactive compounds with minimal side effects *in vivo*.

Ramon is a complex preparation based on natural anthraquinones. It has been educed out of plants by treatment with methanol, reprecipitation with toluene and chromatographic purification on starch.

Ramon appeared to be a long-range antitumor preparation. It produces excellent effect against tumors of human bladder and intestine transplantated to animals so as against wide spectrum of transplantatedsubstrains of animals' tumors being resistant to comparative preparations such as S-fluorineuracil, sarcolysine, rubomycine. Moreover Ramon produces antiflogistic and immunomodulating effects.

Raissa A. Muzychkina is with the Department Chemistry and Chemical Technology, al-Farabi Kazakh National University, Almaty, CO 050040 Kazakhstan (corresponding author to provide phone: 727-387-1751; fax: 727-292-3731; e-mail: rmuz@mail.ru).

Irina M. Korulkina is with the Center for Physical-Chemical Methods of Research and Analysis, al-Farabi Kazakh National University, Almaty, CO 050040 Kazakhstan (e-mail: i.r.k.a.k.i.m@list.ru).

Dmitriy Yu. Korulkin is with the Department Chemistry and Chemical Technology, al-Farabi Kazakh National University, Almaty, CO 050040 Kazakhstan (e-mail: Dmitriy.Korulkin@kaznu.kz).

In comparison with above-mentioned preparations Ramon is safer: when infused in therapeutic doses it doesn't disturb biogenesis of lymphoid cells, functions of liver, kidneys, outer breathing, and cardiovascular system, doesn't provoke thrombocytepenie.

II. Results and Discussion

The antiinflammatory effect of Ramon was studied against asepticinflammation of white rats knee hinge. The preparation was being injected daily for 7 days in the dose 10 mg/kg. Arthritis was caused by 0.l ml turpentine injection into knee hinge [2].

Animals were preliminary perorally treated by Ramon for 7 days while control rats – by water in the equal dose. 34 hours after turpentine injection animals were slaughtered, back limbs were separated out of body in hip hinges, soft tissues were removed, limbs weighed and the growth of knee hinge weight was estimated in percent's in ratio to the healthy one. The resulting weight growth appeared to be average 16.8%for treated by Ramon rats and 38.6%for control animals. It's well seen that the mentioned weight growth was 2.3 times lower in the former case.

The inflammatory edema of all experimental and control rabbits was well seen for two hours and reached its maximum for 24 hours. Therewith sizes increasing of burnt ear concha came to 410.6±35.5% for 24 hours in control and to 160.6±19.4% for rabbits treated by Ramon [see Table I].

TABLE I
Influence of 5% Water Solution of Ramon to the Magnitude of Pinna Edema for Rabbits

Group	Growth of pinna edema (in % to initial after the burn), in hours:		
	2	5	24
Control	135.2±10.2	219.9±21.8	410.6±35.5
Ramon	132.1±22.5	180.0±21.7	160.6±19.4
Group	Growth of pinna edema (in % to initial after the burn), in hours:		
	48	72	96
Control	355.0±37.3	354.9±37.9	322.7±32.6
Ramon	173.2±32.7	175.0±23.3	156.0±16.4

After 96 hours size increasing of ear concha came to 322.7%in control and to 156.0%for treated animals. Necrosis of burnt ear concha was substantially less for treated rabbits. Square of preserved burnt tissues came to 57.8±9.6%in control and to 92.1±3.7%for treated animals.

Experiments for acute toxicity studying were carried out with 176 white mongrel mouses weighing 18 to 20 g, 190

mongrel rats (90 to 140 g), 27 Chinchilla rabbits (2.0 to 2.5 kg), 28 mongrel dogs (5 to 9 kg).

Doses of various lethality levels were found for mouses being injected intraperitoneally with 2%Ramon water solution [see Tables II-IV].

TABLE II
TOXIC DOSES OF 2% WATER SOLUTION OF RAMON-SUBSTANCE ON BEING SINGLY INTRAPERITONEALLY INJECTED TO MOUSES-MALES AND ONES OF ITS MEDICINAL FORM SOLUTION IN 5% GLUCOSE

Doses	mg/kg		Confidence interval	
	water solution	glucose solution	water solution	glucose solution
LD_{50}	610	670	552 to 660	580 to 750

TABLE III
COMPARATIVE STUDY OF RAMON ACUTE TOXICITY WITH MOUSES (MALES AND FEMALES)

Sex	Doses, mg/kg	Lost/total number	Time of loss, days of the trial
Males	200	0/10	-
	300	0/10	-
	400	2/10	10, 13
	500	4/10	2, 3, 4, 5
	600	5/11	2, 3, 4, 4, 6
	700	6/10	1, 2, 2, 3, 4, 5
	900	10/10	1, 1, 1, 1, 1, 2, 2, 2, 2, 3
	1100	10/10	1, 1, 1, 1, 1, 1, 1, 1, 1, 1
Females	300	0/10	-
	400	2/10	9, 14
	500	3/10	2, 4, 5
	600	5/10	1, 3, 4, 4, 5
	700	6/10	1, 1, 2, 3, 4, 4
	900	10/10	1, 1, 1, 1, 1, 1, 1, 2, 2, 2

TABLE IV
TOXIC DOSES OF 2% WATER SOLUTION OF RAMON SOLUTION IN WATER AND IN 5% GLUCOSE WHEN SINGLY INTRAPERITONEALLY INJECTED TO MALES OF WHITE MONGREL RATS

Doses	mg/kg		Confidence interval	
	+	++	+	++
LD_{10}	320	345	-	-
LD_{50}	530	595	502±560	580±630

Not depending on animals species (two ones were studied) loss happened at one and the same time in accordance with the dose: at LD_{90-99} all the animals died in two days, at LD_{20-30}two of ten animals of both species in 10 to 13 days for mouses and 4 to 8 days for rats.

Intraperitoneal injection of toxic doses caused the same clinical picture for the both species. For example at LD_{90-99} activation developed during several hours after injection, then 3 to 5 hours after – hair bristling and depression [3].Beared doses appeared not to influence natural weight growth of mouses and rats' bodies while toxic ones decreased the growth tempo [see TablesV-VI].

TABLE V
INFLUENCE OF RAMON TO MOUSES BODY WEIGH GROWTH

Sex	Dose, mg/kg	Mouses number	Time of experiment					
			5	10	15	20	30	35
Males	400	10	0	+3	+6	+10	+17	+22
	500	10	0	+2	14	+8	+10	+20
	600	11	-2	-7	-1	+4	+7	+11
	700	10	-7	-12	-10	-10	-4	+9
Females	400	10	0	+4	+8	+13	+19	+27
	600	10	0	-6	+1	+9	+9	+19
	700	10	-8	-1	-2	+4	+5	+12

TABLE VI
RAMON INFLUENCE TO RATS BODY WEIGH GROWTH

Doses, mg/kg	Rats number	Time of experiment						
		5	10	15	20	30	35	40
400	10	0	+4	+9	+16	+22	+27	+29
500	10	+3	+6	+11	+15	+19	+24	+27
600	10	-5	-16	-8	+2	+10	+18	+28
700	10	-9	-18	-9	+1	+13	+17	+23

2% Solution was singly intraperitoneally injected in doses 250 mg/kg and 53 mg/kg (LD_{50}) to rats. Beared and toxic doses appeared not to cause at the first, eighteenth and thirtieth days any certain deviations in comparison with control characteristics of blood such as level of erythrocytes, hemoglobin, thrombocytes, lymphocytes, reticulocytes, monocytes, leucocytes (segmental nuclear, rod nuclear and eosinephillic among them) [4]. However mentioned doses gave considerable rise to transaminase activity in blood serum of the same animals and besides the toxic dose caused the growth of residual nitrogen, urea, potassium concentrations and thymol test value not effecting therewith general protein, albumin, globulin, glucose and sodium concentrations (transaminase in first days: control - 0.60±0.01; for 250 mg/kg dose - 0.90±0.03 and for 530 mg/kg dose - 1.20±0.05)[see Table VII].

TABLE VII
INFLUENCE OF RAMON TO BIOCHEMICAL CHARACTERISTICS OF RATS BLOOD SERUM

Dose, mg/kg	Day	General protein, g/l	Albumin, g/l	Globulin, g/l
Control	1	58.0±0.8	28.0±1.0	30.0±1.0
	18	77.6±0.8	33.2±0.3	44.4±0.5
	30	73.6±1.0	32.0±0.9	43.4±1.2
250	1	57.8±2.3	29.2±1.8	38.6±1.8
	18	60.2±1.7	27.0±1.2	33.2±1.5
	30	64.4±1.4	30.0±0.7	34.4±1.0
530 (LD_{50})	1	72.8±0.8	32.0±0.5	40.8±0.7
	18	77.0±1.2	32.0±0.5	40.8±0.7
	30	80.0±2.4	35.2±2.2	44.8±1.6
Dose, mg/kg	Day	Glucose in blood	Potassium mmole/l	Sodium, mmole/l
Control	1	4.4±0.9	38.6±0.9	134.0±1.0
	18	4.45±0.8	35.8±0.8	132.0±1.0
	30	3.7±0.3	48.2±1.3	129.0±2.0
250	1	4.35±0.3	41.6±1.1	128.0±2.1
	18	4.45±0.4	42.0±1.0	126.0±1.5
	30	3.95±0.4	45.0±0.6	127.0±1.0
530 (LD_{50})	1	5.85±1.0	61.0±2.9	128.0±6.1
	18	5.8±1.0	61.0±2.9	128.0±4.6
	30	5.0±2.2	57.6±2.4	131.0±4.7
Dose, mg/kg	Day	Thymol probe, mmole/l	Urea, mmole/l	Residual nitrogen, mmole/l
Control	1	21.6±1.1	4.3±0.2	15.5±0.8
	18	21.8±1.4	4.3±0.2	17.4±1.0
	30	13.0±1.0	4.3±0.5	17.1±0.6
250	1	20.4±1.6	5.5±0.6	22.0±1.3
	18	18.0±1.0	5.0±0.5	20.0±0.5
	30	22.0±1.7	5.6±0.9	26.1±1.0
530 (LD_{50})	1	40.0±2.0	7.1±1.0	28.0±2.5
	18	32.6±1.6	6.6±1.2	26.0±1.8
	30	28.0±1.4	5.9±1.1	24.7±1.4

Moreover bilirubin, lactic and pyruvic acids, general lipids, cholesterol and its ethers, triglycerides, alkaline and acid phosphatase activity level were not effected.

Experiments on effect of Ramon single doses to cardio-vascular system and outer breathingwere carried out with rats and dogs. Ramon 2%water solution was once intraperitoneally injected to rats [see Table VIII].

TABLE VIII
EFFECT OF RAMON BEING INTRAPERITONEALLY INJECTED IN LD_{50} (530 MG/KG) TO WHITE MONGREL RATS-MALES TO ECG AND BREATHING

Time after injection	ECG indexes			
	numeral value			
	R-r per sec.	Systoles frequency per minute	ORT per sec.	syst. index, %
Starting	0.107	561	0.036	33
At once	0.110	545	0.036	33
1 hour	0.112	536	0.037	33
2 hours	0.114	526	0.038	33
3 days	0.112	536	0.037	33
10 days	0.112	536	0.038	34
45 days	0.111	541	0.038	33
Time after injection	ECG indexes			
	% to starting			
	R-r per sec.	R-r per sec.	R-r per sec.	R-r per sec.
Starting	100	100	100	100
At once	104	104	104	104
1 hour	106	106	106	106
2 hours	106	106	106	106
3 days	104	104	104	104
10 days	104	104	104	104
45 days	103	103	103	103
Time after injection	Breathing			
	number of inspirations per minute		% to starting	
Starting	48		100	
At once	50		105	
1 hour	51		107	
2 hours	51		107	
3 days	50		104	
10 days	50		104	
45 days	49		102	

In time period from zero to 45 days Ramon appeared not to change breathing and cardiac frequency and electrocardiogram characteristics.

Ramon 10%water solution was singly injected to femoral vein of animals under morphine-hexenale narcosis (1 ml in 20 to 30 seconds) at 20, 25, 30 and 40 mg/kg doses. 30 minutes after narcosis a dog was fixed at the operating table. Common carotid was separated; Ludwig manometer was connected with its central part to put down arterial tension by kymograph [3]. Solutions were injected through canula put into the femoral vein. Blood coagulability was prevented by 3 mg/kg intravenous injection of heparin 1%water solution. Fluctuations of arterial tension, pulse oscillations amplitude, systoles and breathing frequency were measured while the starting data were considered to be the control ones.

Arterial tension of dogs was not considerably effected by Ramon on being injected in doses 20 and 25 mg/kg. The preparation exhibits positive inotropic effect increasing systoles strength per 25 to 37% in comparison with starting point. Toxic and lethal doses of Ramon (30 to 40 mg/kg) when intravenously injected decreased arterial tension of dogs per 40 to 90%therewith amplitude of pulse oscillations reduced and systoles rhythm rated. Growth of breathing frequency and its strength appeared to be a response to the injection of toxic doses.

Electrocardiogram analysis showed that systole value at the end of the trial after 20mg/kg Ramon injection coincides the starting dispersal bound estimated in accordance with Bazett

formulae. Fluctuations of dogs' chambers electric systole depending on time were slight in comparison with control. Pathologic shifts of peaks and intervals were absent. Some lengthening of R-P and T-R intervals because of breathing arrhythmia was noticed in the only one case [5]. When Ramon was injected in the dose 25 mg/kg as time of chamber electric systole as systole value of dogs still remained normal. Cardiac rhythm didn't change. Once only there was a slight widening and deepening of T- and R-peaks.

Deepening and stratifying of T- and R-peaks were observed for all dogs at the moment and straight away after injection of 30 mg/kg Ramon. Cardiac rhythm and cardiac complex were completely disturbed in 1.5 to 2 minutes. Animals died respectively on the first, fifth, fourth, tenth and sixteenth minute after the treatment.

Ramon when injected in the dose 40mg/kg leads to lethal result on the third, fourth and sixth minutes due to substantial edema of lungs and distinct filling of inner organs with blood.

The foregoing means that Ramon on being once intraperitoneally injected to the dogs in doses 20 to 25 mg/kg doesn't substantially influence arterial tension and breathing. When doses are subtoxic and lethal (30 to 40 mg/kg) arterial tension sharply falls.

So, therapeutic doses of Ramon don't cause any changes in cardiovascular activity and breathing of animals while the toxic ones lead to disturbance of these functions for dogs but not for rats.

Ramon solution in water and 5%glucose was singly injected into bladder of intact rats in doses 250, 500 and 1000 mg/kg. The control rats were injected with the same volumes of water mixed with 5%glucose. Each group consisted of 5 animals. Substantial and lyophilized medicinal forms of the preparation were both tried. General status and survival of rats were observed for 30 days.

The dose 250 mg/kg cause neither loss nor deviations in status and behaviour. Autopsy demonstrated no changes as in viscera as in bladder urinary. Pathomorphological study didn't reveal any deviations in comparison with control.

The dose 500 mg/kg resulted in 20%rats' loss. Hardening in bladder region was absent under palpating. Autopsy revealed moderative duration of bladder walls blood vessels. Morphological study didn't reveal substantial deviations as viewed from viscera as from bladder in comparison with control. Ramon dissolved in 5%glucose didn't cause rats loss in the mentioned dose.

The dose 1000 mg/kg resulted in 40%rats' loss. Palpation revealed hardening in the zone of bladder urinary. Autopsy demonstrated blood vessels of bladder being strongly injected and bladder itself being wrapped from the outside by mesenteries of thin intestine. Morphological study revealed no pathology in the sites of rats' bladder walls.

Loss of animals caused by intolerable doses of Ramon occurred on the eighth-tenth day after injection. Toxicose and disuria developed slowly and were due to intensive irritating effect towards the mucous membrane of bladder. Imperfection of bladder lining was observed as well. The phenomena were less evident when 5%glucose was used.

Atonies, loss of appetite, general oppression were the symptoms of intoxication ran out in 15 to 20 days and subsequently normalized.

Control rats revealed neither loss nor behaviour and status deviations. Autopsy also exhibited no variations viewed as from viscera as from bladder.

10%Solution of Ramon-lyo was singly injected into rabbit's urinary bladder in doses 1000 and 2000 mg/kg. Every dose was tried on 5 males and 5 females. Control animals (5 males and 2 females) were injected with the identical dose of water.

Study of general status and survival was carried out for 40 days. Mentioned doses didn't cause loss of animals and deviations in behaviour. Autopsy revealed no variations viewed as from viscera as from bladder.

Acute toxicity of Ramon-lyo was studied with regard to 12 mongrel adult dogs (6 males and 6 females). 10% Solution in water and 5%glucose heated to 36 to 37 was singly injected into dogs bladder in the dose 1000 mg/kg (4 males and 4 females) and 2000 mg/kg (4 males and 4 females). Control group consisted of 4 males and 4 females being injected with identical doses of sterile distilled water [6].

Study of general status and survival was carried out for 45 days. Mentioned doses didn't result in loss of dogs or deviations in behaviour, status body weight in comparison with control. Autopsy revealed no variations viewed as from viscera as from bladder.

Trials were carried out with 40white mongrel mouses weighing 20 to 24g. Animals were put alone into glass funnels with a screen the ends of which were let down into calibrated test-tubes. The first group of animals was preliminary intraperitoneally injected with 0.5 ml of distilled water and the second one – 45%glucose. Treated mouses were moreover singly intraperitoneally injected with 100 to 300 mg/kg of Ramon. 0.5, 1, 2, 3, 4, 5 and 24 hours after the urine was gathered by micropipette and the results were compared with the control ones. Control and experimental mouses appeared to secret respectively 0.54 to 0.57 and 0.57 to 0.60 ml of urine. Thus Ramon didn't cause substantial diuresis increasing in comparison with control (P). Maximal increasing of the urine amount (0.59 to 0.60 ml) was marked within 4 hours.

Diuresis of rabbits (5 males and 5 females) and dogs (6 males and 4 females) on being treated with 150 mg/kg and 250 mg/kg of Ramon solution in water and 5%glucose respectively was within the normal and starting data limits.

Study of Ramon hepatotoxic effect has been carried out by a method which based on the Ramon ability to destruct Nembutal [1]. Functional state of liver was evaluated according to its histology and sleep duration of mouses treated by Nembutal and Ramon. Being injected intraperitoneally in doses 10, 30, 80 and 160 mg/kg Ramon produces rendering harmless effect (experimental mouses awake earlier than control ones: in 35 to 90 minutes). For example sleep duration for mouses treated by the preparation in the dose 80 mg/kg was 95 minutes after 3 days and 132.3 minutes for control ones. So, Ramon produces antitoxic effect to liver.

III. Experimental

Histochemical methods used: revealing of DNA-granules according to Felgene therewith preparations without preliminary hydrolysis worked as reaction control; RNA revealing – by the Brasche method therewith sheares were treated by ribonuclease to control the reaction; neutral lipoids revealing – by colouring with sudan III [3].

Cutted pieces of tumor tissue were fixed in neutral formalin, Karnua and Buen liquid and poured over paraffin. Sheares were coloured by hematoxiline-eosine, collagen fabers – according to van-Gizone, impregnation of argirophilicfabers – by the Foot method [7].

*Antiinflammatory activity.*Ramon was locally used in trials with 10 rabbits (weighing 2.5 to 3.0 kg) having thermic burn of the ear as a centre of inflammation. Animals were dynamically tested concerning local temperature, number of leucocytes and erythrocytes, hemoglobin. The thermic burn of the ear's concha distal part resulted from 3-minute plunging of the ear to the water bath heated to 53 C. Ears of 5 rabbits were then treated by Ramon, ears of 5 control animals – by distilled water according to the following scheme: right after the burn, 3 to 5 hours after it twice a day for 5 days. Intensity of edema and size of vulnerable tissues necrosis served as criteria of inflammatory reaction. Intensity of edema was determined in accordance with Saljamov method. In order to find percent of the preserved tumor the contour of the ear part ought to be burned was put down to the graph-paper.

After necrosised areas splitting the contour of preserved zones of burnt tissue was marked. In both cases square was evaluated. All figured data were handled with t-criterion [2].

*Macroscopic study.*Liver, spleen, lungs of lost mouses, rats and dogs when studied macroscopically demonstrated plethora, blood vessels of abdominal cavity – hyperemia, intestines – tympania [4].

References

[1] M.Hidalgo, E.Garrett-Mayer, N.J.Clendeninn, S.G.Ekhardt, *Principles of Anticancer Drug Development*, New York: Springer, 2011.
[2] J.Y.Chang, A.J.Lewis*Pharmacological Methods in the Control of Inflammation*, Wiley-Blackwell, New-York, 1989.
[3] S.K.Gupta, *Drug Screening Methods*, Kent: Anshan Publishers, 2009.
[4] A.A.Adjei, J.K.Buolamwini, *Novel Anticancer Drug Protocols*, New York: Academic Press, 2011
[5] H.G.Vogel, J.Maas, A.Gebauer, *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, Berlin: Springer, 2011.
[6] A.A.AlFattah Amara, "Methods other than Experimental Animals for Screening Antitumor Compounds," *Am. J. Cancer Sci.*, vol. 2, no. 1, pp. 1-27, 2013.
[7] J.Kolman, P.Meng, G.Scott,*Good clinical practice: standard operating procedures for clinical researchers*, New-York:J. Wiley & Sons, 1998.