

Studies on potential mutagenic and genotoxic activity of Setarud

^{1,2} Khorram Khorshid H.R., ³Novitsky, Y.A. ⁴Abdollahi M., ⁵ Shahhosseiny M.H.,
⁶Sadeghi, B. ⁷Madani H., ³. Rahimi R, ^{*8}Farzamfar B.

¹Genetic Research Centre, Social Welfare and Rehabilitation Sciences University,
²Reproductive Biotechnology Research Centre, Avicenna Research Institute (ACECR), ³Pars
Roos Pharmaceutical Co., ⁴Faculty of Pharmacy, and Pharmaceutical Sciences Research
Center, Tehran University of Medical Sciences, Tehran, ⁵Department of Microbiology,
Shahryar-Shahr-e-Qods-Branch, Islamic Azad University, Iran, ⁶Experimental Cancer
Medicine, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden,
⁷Department of Biotechnology, Rabe Rashidi Institute, Tabriz, ⁸Pasteur Institute of Iran,
Tehran, Iran

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ABSTRACT

Background: Setarud (IMOD™) is a new herbal drug that has demonstrated immune modulating activity in preliminary investigations. The aim of this study was to evaluate the potential of mutagenicity and genotoxic properties of Setarud following the guidelines of the Organization for Economic Co-operation and Development (OECD) for the Testing of Chemicals.

Methods: Ames *Salmonella*/mammalian microsome mutagenesis assay was used to evaluate the ability of the drug and its metabolites to induce mutation in *Salmonella* tester strains. Setarud was applied in concentrations of 0.1-1000 µg/dish. The effect of the drug metabolites which were formed in the presence of rat liver microsomal fraction S9 was investigated using complete and incomplete microsomal activation mixtures, separately. Induction of dominant lethal mutations in spermatogenic stem cells of male mice was also assessed.

Results: In the Ames test, the drug preparation did not cause a significant increase in the number of revertant bacterial colonies as compared with negative control meaning that Setarud within the tested range did not exhibit mutagenic activity. The level of post-implantation losses and as a result the number of lethal mutations in germ cells at different stages of spermatogenesis in mice treated with Setarud was not statistically higher than that of control.

Conclusion: Under experimental conditions which were employed, the drug was not mutagenic or genotoxic.

Keywords: Setarud, IMOD™ drug, Genotoxicity, Mutagenicity, Dominant Lethal Mutations

INTRODUCTION

In the search for new drugs with the capacity to correct immune deficiencies, a compound "Setarud" a mixture of herbal extracts (*Tanacetum vulgare*, *Rosa canina*, *Urtica dioica*) supplemented with selenium was prepared. Due to the nature of its ingredients, Setarud exerted positive effects on the immune system, serum lipid profile, and showed hepatoprotective activity in animals (1-2). Of the drug constituents, *T. vulgare* (tansy) is a useful remedy for treatment of a wide range of symptoms due to anti-inflammatory effects (3). Beta-caroten of the

fruits of *R. canina* delays the increase in serum cholesterol and blood glucose (4). Polysaccharide and lectin fractions of *U. dioica* (nettle) are shown to have anti-inflammatory and anti-viral activities (5,6). In addition, potentially toxic properties of tansy (7) and poisonous effects of nettle lectins (8) have been documented.

Selenium is an essential trace element for both human and animals which plays a key role in protecting cells from oxidative stress (9-10). Se supplementation in the diet may reduce the risk of cancer, cardiomyopathy and immune disorders in humans (11-14). However, Selenium toxicity can

occur in mammals from either acute or chronic exposure (15).

For determination of mutagenic potential of Setarud Ames *Salmonella* reverse mutation test was employed. In this test employs histidine-dependent tester strains is used to detect mutations, which involve substitution, addition or deletion of one or a few DNA bases reverting originally changed gene sequence of the tester strains. This restores their functional capability to synthesize histidine and to grow in the absence of the amino acid required by the parent strain (16). The genotoxicity of the drug was carried out in rodent dominant lethal (DL) mutations assay to determine potential mutagenic and genotoxic effects of Setarud in indicator microorganisms and animals.

MATERIALS AND METHODS

Chemicals and reagents

Standardized Setarud extract (IMOD™) was supplied by Pars Roos Co. (Tehran, Iran). A fresh stock of the drug was prepared in distilled water to a final concentration of 10 mg/ml. 4-nitroquinoline 1-oxide (4NQO), 9-aminoacridine (9AA), sodium azide (NaN₃), 2-aminoanthracene (2AA), and Aroclor 1254 were purchased from Sigma-Aldrich were used.

Experimental animals

CBA and C57Bl/6J mice weighing 18-20 g were purchased from Karaj Breeding Center (Tehran, Iran) and assigned to tests following 5-7 days acclimation. The animals were kept in T-3 type cages at 20-22°C and 60-65% humidity under a 12-hrs light/dark cycle and had free access to food and water supply.

Bacterial reverse mutation test (Ames test)

Histidine auxotrophic strains of *S. typhimurium* TA98, TA100, and TA1537 (from Experimental Cardiology Institute, Ministry of Health of Russian Federation) were pretested for auxotrophicity, the presence of plasmid pKM101 and rfa mutation and then used in the assay. The stock of Setarud was serially diluted and applied to Petri dishes in concentrations of 0.1-1000 µg/dish. Well-known mutagens such as 4-nitroquinoline 1-oxide (4NQO), 9-aminoacridine (9AA), and sodium azide (NaN₃) were used as positive control substances. Distilled water was used as a negative control. Cofactor-supplemented post-mitochondrial supernatant of homogenate (S9 fraction) prepared from the livers of 7 week old male Sprague-Dawley rats treated with Aroclor 1254 was employed as metabolic activation system. 2-Aminoanthracene (2AA), as

standard for indirect mutagen requiring metabolic activation was applied in appropriate experiments (16).

For performing the Ames test, fresh cultures of bacteria were grown under suitable conditions, at 37°C up to the early stationary phase of growth (approximately 10⁹ cells per ml). Then, selective semi-concentrated agar was melted in the test tubes and cooled in water bath to 44-45°C. One hundred µl of appropriately diluted Setarud or negative and positive controls were mixed with 100 µl of bacteria culture suspension, 100 µl S9 rat liver fraction, cofactors and then combined with the melted agar. The mixtures were blended and immediately poured on the layer of minimal agar in a Petri dish. After complete solidification of agar, Petri dishes were incubated at 37°C. After 48 hrs of incubation, the number of prototrophic revertant colonies in each plate was counted.

To investigate the action of the drug metabolites which formed in the presence of S9 fraction, tests were repeated 3 times with different concentrations of the tested compounds and complete (CMAM) or incomplete microsomal activating mixture (IMAM). The mutagenic frequency was expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. A mutagenic potential of a test substance was assumed if the mutant frequency was 2.0 or higher. A possible mutagenic potential could also be supposed if the quotient ranged from 1.7 to 1.9 in combination with a dose-effect relationship.

Dominant lethal mutations in mice

Setarud at a dose of 21 mg/kg body weight diluted (1:10) in normal saline (treatment group, n=12) or the same volume of normal saline (control group, n=12) were injected as single dose I.M. to 8-10 week old CBA males. Then, each male was housed with 3 intact virgin C57BL/6J (C57) females, which were replaced by new ones at 7-day intervals for a total of three 7-day breeding periods. This mating scheme provided data for the analysis of all stages of mice spermatogenesis. On days of 15-17 of pregnancy, all females were sacrificed by cervical dislocation for uterine evaluation. The total number of implants, number of live implants, number of early resorptions or moles (dead implants), and late deaths were recorded at the time of dissection. DL effects were calculated on the basis of the average number of live implants in the treated group versus the number of those in the control group (16).

Statistical Analysis

Student's *t* test was used to compare means. All measurements were taken to enable calculation of

Table 1. Effect of Setarud on Salmonella indicator strain TA98 in the Ames test

The studied substance	Dose $\mu\text{g}/\text{dish}$	TA98 strain							
		-S9				+S9			
		Mi	M	Mo/Mk	MA	Mi	M	Mo/Mk	MA
Control H ₂ O	0	33	32.8		-	40	35.5		-
		29				36			
		37				31			
2AA	20					1211	1209	34.0	+
						1280			
						1140			
4NQO	0.5	568	582.6	17.8	+				
		567							
		614							
	0.1	33	34.0	1.04	-	30	38.4	1.08	-
		32				42			
		35				45			
	1.0	29	32.5	0.99	-	40	34.4	0.97	-
		32				35			
		37				29			
Setarud	10.0	28	33.1	1.01	-	36	34.3	0.97	-
		34				35			
		38				32			
	100.0	27	27.6	0.84	-	19	25.1	0.71	-
		30				27			
		26				31			
	1000.0	29	29.9	0.91	-	30	32.0	0.90	-
		28				28			
		33				39			

Abbreviations: **Mi**: the number of revertants per dish, **M**: average geometrical number, **Mo/Mk**: the ratio of the number revertants in test to the number of revertants in control, **MA**: mutagen activity, **2AA**: 2-aminoanthracene, **4NQO**: 4-nitroquinoline 1-oxide, +: the presence of activity, -: the absence of activity

the mean and SD for the outcome variables. In the Ames test, data were analysed with the SALANAL (Salmonella Assay Analysis, version 1.0, Research Triangle Park, NC, USA) software applying the Bernstein model (16). *P* value of <0.05 was considered as statistically significant.

RESULTS

Bacterial reverse mutation test

In the Ames test, the number of revertants induced by different amount of Setarud (0.1-1000 $\mu\text{g}/\text{plate}$) for all tester strains were close to those of the negative controls and much lower than those of positive controls. No mutagenic activity for the test substance was observed in tested concentrations with or without S9 mix. All the quotients ranged between 1.0 (or lower) and 1.6 (Tables 1-3). No dose-effect relationship was observed.

Dominant lethal mutations in mice

The genotoxicity data of Setarud in male mouse germ cells is demonstrated in table 4. It indicates that the level of post-implantation losses in females mated to males, which received a single

intramuscular injection of Setarud (21 mg/kg or 0.7 ml/kg) was not higher than that of controls.

DISCUSSION

Due to close correlation between mutagenicity and carcinogenicity, finding a mutagenic potential predicts its carcinogenicity. Different methods have been utilized which have their own advantages and limitations and no method which can detect all the mutagenic compounds has been identified. In fact, results of the various methods for detection of mutagenicity can not fully applied to humans as they have special defensive and repairing systems.

From various methods currently used for detection of mutagenicity of compounds, the revertant *Salmonella typhimurium* species used in Ames test is one of the most frequent used procedures (16). A comparison of the various mutagenicity methods demonstrated that Ames test has special features including good sensitivity and specificity. Therefore, despite false negative results, Ames test has been used as a screening test for compounds with a high sensitivity and specificity.

Table 2. Effect of Setarud on Salmonella indicator strain TA100 in the Ames test

The studied substance	Dose $\mu\text{g}/\text{dish}$	TA100 strain							
		-S9				+S9			
		Mi	M	Mo/Mk	MA	Mi	M	Mo/Mk	MA
Control H ₂ O	0	144	194.2			188	223.2		
		223							
		228							
2AA	20					2712	2288.2	10.2	+
Sodium azide	2.0	1240	1297.6	6.68	+				
		1368							
		1288							
Setarud	0.1	185	185.8	0.96	-	190	239.0	1.07	-
		154							
		225							
	1.0	154	177.0	0.91	-	176	193.3	0.87	-
		169							
		213							
10.0	153	195.2	1.00	-	175	184.2	0.82	-	
	201								
	242								
100.0	165	198.0	1.02	-	146	181.0	0.81	-	
	221								
	213								
1000.0	209	206.0	1.06	-	131	151.2	0.68	-	
	207								
	202								

Table 3. Effect of Setarud on Salmonella indicator strain TA1537 in the Ames test

The studied substance	Dose $\mu\text{g}/\text{dish}$	TA1537 strain							
		-S9				+S9			
		Mi	M	Mo/Mk	MA	Mi	M	Mo/Mk	MA
Control H ₂ O	0	6	7.11			9	9.62		
		6							
		10							
2AA	20					126	128.9	13.4	+
9AA	2.0	5448	5609.3	788.5	+				
		5632							
		5752							
Setarud	0.1	6	5.24	0.74	-	7	6.95	0.72	-
		6							
		4							
	1.0	8	7.32	1.03	-	10	8.62	0.90	-
		7							
		8							
10.0	10	9.65	1.36	-	5	3.56	0.37	-	
	10								
	9								
100.0	5	5.00	0.70	-	8	7.96	0.83	-	
	5								
	9								
1000.0	10	8.96	1.26	-	7	5.94	0.62	-	
	8								
	9								

Table 4. The influence of Setarud on the induction of dominant lethal mutations in mice

Stage of spermatogenesis	Setarud Dose (mg/kg)	Total number of female	No. of pregnant females	Fertility %	Post-implantation losses
Mature sperm	0	36	29	81	0.053
	21	33	29	88	0.044
Late spermatide	0	36	32	89	0.068
	21	35	28	80	0.066
Early spermatide	0	36	32	89	0.062
	21	33	28	85	0.056

In this study, mutagenic potential of Setarud was evaluated for the first time. In the Ames test, it was observed that the number of revertant colonies in *S. typhimurium* strains did not increase in the presence of the drug. Thus, Setarud and Se as one of its main components do not display any mutagenic properties that is supported by an earlier report (17).

The dominant lethal test detects mutations which cause the death of rodent embryos. The usual procedure in this test is to treat male mice with a suspected mutagen and then mate them with untreated females. If the treatment causes a mutation in a male germ cell that is lethal at some stages of the development of the embryo (after fertilization), the lethal events are detectable by dissecting female uteri a few days after implantation of embryos (implantation occurs about nine days after fertilization). If there are more dead embryos than expected (comparison with matings of unexposed mice) and/or more

implantation sites than expected in the uterus where there is no longer an embryo, the effect is assumed to be the result of a mutation in the male germ cell. Chromosomal studies in mice have shown that almost all "dominant lethal" events are associated with major chromosome abnormalities. In dominant lethal mutations in mice, Setarud at tested dose did not increase the number of lethal mutations in male germ cells at different stages of spermatogenesis and showed no genotoxic effects.

CONCLUSION

From the data obtained in both tests it may be concluded that setarud (IMOD™) had no mutagenic or genotoxic activities at the dose ranges which were used in the test.

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