Preclinical and phase 1 clinical safety of Setarud (IMOD™), a novel immunomodulator

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Received 2 Feb 2009; Revised 1 July 2009; Accepted 12 July 2009

ABSTRACT

Background: A new herbal drug, Setarud (IMOD™) that has been shown to have beneficial immune effects was tested to determine its acute and chronic toxicity in animals and to establish its intravenous form maximum tolerated dose (MTD) in an open-labeled phase I clinical trial.

Methods: BALB/c and C57BL/6 mice and Wistar rats were monitored for general state and biochemical markers for chronic test. At the end of chronic test, animals examined macroscopically and histologically. HIV-infected asymptomatic male patients with CD4 counts more than 200, were enrolled in the trial. Baseline dose was calculated from the 10% lethal dose (LD_{10}) established in laboratory animal studies. Dose escalation was performed in four cohorts of 3 patients receiving IMOD™ intravenously at a cohort-specific dose of 2, 4, 6.7, and 10 ml daily for 4 weeks. Patients were clinically examined at days of 1, 2, 3 and then weekly; and the safety was assessed on the basis of reports of adverse events, laboratory-test data and toxicity signs.

Results: LD_{50} values in acute toxicity test were 42-66 and 50-56 ml/kg in i.m. and i.p. injections, respectively. Total scores of embryotoxicity during pregnancy were significantly lower in the Setarud group ($p < 0.05$). Pre-implantational deaths in the Setarud group were significantly higher, but post-implantational deaths level was lower than those in the control group. Inhibition of ossification in the skeletons of the fetuses and incidence of still birth were significantly higher while body weight of new-born rats of treatment group in the first month of their lifes were lower than those of the control group.

In all four cohorts, there were no major side effects or dose-limiting toxicity, except for a mild sweating and weight loss in two patients from the first group that was reversed without discontinuation of the treatment. No clinically relevant trends in laboratory test results or ECG changes were noted. No adverse effect due to IMOD™ was observed at one month of follow-up. Maximum tolerated dose of IMOD™ was 10 ml a day.

Conclusion: Results of this study has identified a safe dose of IMOD™ that can be used in future clinical trials.

Keywords: Setarud, IMOD™, Acute toxicity, Chronic toxicity, Maximum Tolerated Dose

INTRODUCTION

A new drug "Setarud" with the capacity to correct immune deficiencies was introduced for the treatment of human immunodeficiency virus (HIV) infection (1). Being a mixture of herbal extracts (*Tanacetum vulgare, Rosa canina,* and *Urtica dioica*) enriched with selenium (Se), Setarud demonstrated positive effects on the immune system when administered to animals. Herbal ingredients of the drug may exert anti-inflammatory, anti-viral and immune-

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stimulating properties (2-4). Being rich in beta-caroten, polysaccharides and lectins, these herbs are potentially useful for treatment of a wide range of complaints including high serum cholesterol and blood sugar levels (5, 6). Selenium is an essential trace element that plays a key role in protecting cells from oxidative stress (7-10). However, excessive Selenium intake is toxic in mammals (11).

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV) and is one of the world's serious health problems (12). HIV is a retrovirus which damages the immune system by targeting CD4 lymphocytes and patients become more susceptible to opportunistic infections (13).

Highly Active Retroviral Therapy (HAART) regimen is the principal management strategy for patients with HIV infection (14). Several side effects of HAART have been reported including life threatening conditions such as lactic acidosis, hepatitis, drug reactions and cardiovascular disease (15-16). In addition to multiple side effects, increasing resistance to HAART regimen in the last decade and transmission of multi-drug resistant strains has critically restricted the effectiveness of this regimen (17-19). Furthermore, cost of HAART is an important obstacle, limiting access to these drugs especially in developing countries (20). Complexity of the regimens, poor tolerability, metabolic side effects, toxicities and high cost are some of the reasons for poor adherence.

Immune-based therapy is a new concept in the treatment of HIV-infected patients which has been developed in the past few years. Immune therapy using vaccines, cytokines and hormones is based on stimulation of immune response in HIV-1 infected individuals, leading to immune control of virus replication (21). Such response has been previously observed in untreated HIV-1 infected long-term non-progressor (LTNP) patients as well as some other viral diseases (22, 23). Many drugs from this class of agents are being assessed in clinical trials, but none have yet been approved for use in HIV infected patients. Setarud is a novel herbal extract which is proposed to have immune-system stimulating properties. Preliminary studies have indicated safety and efficacy of Setarud in laboratory animals (24-26) but on the basis of regulations for a new drug approval it is necessary to complete safety studies. In the present work, Setarud was tested for acute and chronic toxicities in animals and went through phase I clinical trial in HIV-infected patients to determine maximum tolerated dose (MTD) and dose limiting toxicity (DLT).

MATERIALS AND METHODS

Preparation of Setarud (IMOD)

Leaves and small stems of *U. dioica* and *T. vulgare* and fruits of *R. canina* were dried in a dark place for 3-4 days at 42°C and broken into small pieces. Then, they were separately packed into glass vessels and extracted with 96% ethanol at 42°C for 30 days (1). Extracts of green and orange-red colour were separated from material using a cloth filter. Then, 30 ml of *R. canina* and one liter of *U. dioica*/*T. vulgare* extracts were enriched with 16 mg of Se. After 24 h incubation at 42°C, the mixture was sequentially passed through a millipore 5.0, 0.45 and 0.22 µm filters, and dispensed into sterile vials for research use under the trademark name of IMOD (Pars Roos Co., Tehran, Iran). Each vial was set to contain 125 mg of active ingredients in 4 ml solution.

Animals

Male and female BALB/c, CBA and C57BL/6J (C57) mice with body weight (b.w.) of 18-20 g and Wistar rats (180-220 g) were purchased from Karaj Breeding Center (Iran), housed for two weeks before experiments in a temperature and light-controlled room with a 12-hrs light/dark cycle and provided with food and water ad libitum until one day prior to experimentation. All ethical issues on the use of animals were carefully considered and each experiment was approved by the institute review board.

Determination of LD_{50} and acute toxicity study

One-hundred twenty-eight BALB/c mice and 47 Wistar rats were separated into groups and injected intramuscularly (i.m.) or intraperitoneally (i.p.) with Setarud (diluted at 1:5 ratio in sterile saline) at dosages of 0 (controls), 750, 1500, 2250 and 3000 ml/kg. The animals were observed for mortality, morbidity and signs of toxicity for 30 days. LD_{50} was determined using the probit-analysis (27).

Chronic toxicity

Ninety Wistar Rats were divided into 3 groups, 15 males and 15 females each, received once a day i.m. sterile saline (the first, control group) or 2.1 and 6.3 mg/kg b.w. Setarud for the second and third groups, respectively, during 3 months. General state and behavior of the animals including body weight, rectal temperature, motor activity, appetite and hair condition were registered. At the beginning and after 1 and 3 months of treatment, hematological and biochemical markers of animals were determined by collecting 2-2.5 ml of blood from caudal vein of rats. At the end of chronic experiment, euthanasia of rats by overdosage of diethyl ether was performed for the purpose of pathomorphological examination of their inner organs and tissues. Organs were removed and fixed with 10% buffered formalin for 24-48 hrs, then cut with a razor blade and embedded in paraffin. The paraffin-embedded tissues were cut into 5 µm sections, stained with hematoxylin and eosin, and

Table 1. Patients characteristics (n=12).

Arm	Dosing Schedule (mL of 125 mg/4 mL sol.)	Age (year)	CD4 count (cell/μL)	HIV RNA level (copies/mL)
A	2	47	420	21600
A	2	37	831	6180
A	2	27	437	91900
B	4	30	597	227000
B	4	50	389	306000
B	4	34	367	1740
C	6.7	28	263	155000
C	6.7	43	367	56300
C	6.7	42	312	166000
D	10	34	620	75300
D	10	60	429	1680
D	10	34	363	13200

Table 2. Utilized doses of Setarud.

Dose	Explanations
2 ml	Based on one tenth of LD_{10}
4 ml	Based on 100% increase of the first dose
6.7 ml	Based on 67% increase of the second dose
10 ml	Based on 55% increase of the third dose

used for histopathological examinations according to standard methods.

Embryotoxicity and teratogenicity assay

Of 60 female and 30 male Wistar rats, each two females were placed together with one male for mating. Females showing evidence of mating (day 0 of gestation) were randomly assigned to control and treatment groups up to the day when 18 females had been allotted to each group. The treatment and control groups received i.m. Setarud 6.3 mg/kg daily and the same amount of sterile saline, respectively, during all period of pregnancy. The animals were observed twice daily for signs of toxicity and their body weights were determined on days 0, 7, 14, and 21 of gestation. On the day of 21 of gestation euthanasia of 70% of pregnant rats was carried out by means of dislocation of cervical vertebrae with subsequent examination of the bony skeletons and internal organs of the fetuses and determination of the indices of pre-implantational and post-implantational deaths according to the formulas:

Pre-implantational death % = (the number of yellow bodies-the number of implantation sites) × 100 / the number of yellow bodies

Post-implantational death % = (the number of implantation sites – the number of alive fetuses) × 100 / the number of implantation sites

After evaluation of viability of fetuses and determination of their weights, external anomalies and developmental defects were evaluated. Then 2/3 of the randomly selected fetuses were eviscerated, partly skinned, fixed in 70% ethanol, cleared in potassium hydroxide, and stained with alizarin reds for examination of the skeleton (28). The remaining fetuses were fixed in bouin's fluid for subsequent visceral examination according to the Wilson technique (29). The urinary bladder, urethra and reproductive organs were also investigated. In 30% of total pregnant females that were not killed the lethality, body weight, and physical development of new-born rats were investigated after delivery.

Human studies

Twelve HIV-infected asymptomatic male patients were enrolled into the study. Inclusion criteria were age between 18 and 65, having two positive ELISA and one positive Western Blot test result, CD4 counts more than 200, and no history of previous anti-HIV or immune therapy treatments. Patients with current or history of malignancy or severe diseases such as renal or hepatic failure, chronic heart failure, advanced eye disease, opportunistic infections such as Pneumocystis carinii pneumonia (PCP) or other severe infections, abnormal liver function tests, bilirubin level more than 1.5 times of the upper normal limit, were excluded (Table 1).

Ethical considerations

On enrollment, all patients were informed of study objectives, probable adverse effects of Setarud and their rights if they decide to join the study. Participation was completely voluntary and patients were free to leave the study at any stage. Ethical approval was obtained from Ethics Committee of Tehran University of Medical Sciences. Patients were informed that all costs related to management of any probable adverse effects will be covered by study sponsor and their transport costs will be re-imbursed.

Study design

The trial was designed based on modified Fibonacci

Table 3. Liver enzyme levels in male rats during 3-month i.m. injection of Setarud.

	Control	Setarud	
		2.1 mg/kg/day#	6.3 mg/kg/day#
Males			
Alkaline phosphatase, unit/l			
Before injection	464.8 ± 34.2	492.4 ± 28.4	506.8 ± 26.8
1 month	460.8 ± 27.4	475.6 ± 42.2	447.4 ± 63.1
3 month	490.2 ± 24.2	500.2 ± 24.2	484.1 ± 26.5
Alanin aminotransferase, unit/l			
Before injection	67.87 ± 4.66	62.81 ± 6.43	60.42 ± 4.67
1 month	67.01 ± 6.65	66.60 ± 3.60	60.80 ± 5.96
3 month	62.88 ± 4.22	68.37 ± 5.08	65.73 ± 6.33
Aspartate aminotransferase, unit/l			
Before injection	92.37 ± 9.28	98.38 ± 9.41	96.52 ± 6.48
1 month	84.21 ± 8.89	80.99 ± 5.49	92.63 ± 9.79
3 month	88.98 ± 4.29	92.13 ± 9.87	88.60 ± 5.16
Lactate dehydrogenase, unit/l			
Before injection	847.28 ± 67.18	869.84 ± 48.36	848.36 ± 67.28
1 month	826.25 ± 62.80	907.60 ± 74.56	892.29 ± 62.05
3 month	858.68 ± 64.24	918.23 ± 61.15	897.86 ± 64.46

not statistically significant (p>0.05)

Table 4. The indices of embryotoxic effect of i.m. Setarud injection (6.3 mg/kg) during gestation.

	Control	Setarud
Duration of pregnancy (days)	24.5 ± 0.2	23.3 ± 0.2
Number of embryos per rat	9.5 ± 1.3	5.5 ± 0.6*
Number of implantation sites per rat	10.0 ±1.2	6.0 ± 0.8*
Number of yellow bodies per rat	11.5 ±0.5	8.9 ± 0.8*
Pre-implantational death (%)	13.5	40.8 *
Post-implantational death (%)	8	5.1
Cranio-caudal size of fetus (cm)	3.3 ± 0.1	3.0 ± 0.1
Weight of fetus (g)	3.0 ± 0.1	2.2 ± 0.2*

* Statistically significant ($p < 0.05$)

dose escalating method as depicted in (Figure 1). Patients were included in cohorts of 3 individuals and the administered doses were equal in all 3 members of each cohort. Baseline dose was calculated as 10% of the lethal dose (LD_{10}) established in laboratory animal studies. Patients of the first cohort were treated with 0.03 ml/kg of Setarud (125 mg per 4 ml solution) diluted in 100 ml normal saline and infused over 30 minutes. Dose escalation was performed according to modified Fibonacci method. Patients in the subsequent groups received 4, 6.7 and 10 ml of Setarud, respectively (Table 2). Treatment was continued for 28 days for the patients in each cohort. The study progressed to the next dose in the absence of DLT manifestation after 28 days. Common toxicity criteria scale published by National Cancer Institute for assessment of DLT (30) and grade III adverse events published by WHO (31) was used in this study.

Response assessment

A complete set of clinical and para-clinical assessments were performed to monitor possible adverse effects of Setarud in patients with HIV infection. All laboratory evaluations were carried out in Iranian Research Center for HIV/AIDS (IRCHA). CD4 was counted using Deko (Denmark) and viral load was measured by Cobas Amplicore (Roche). A baseline assessment was performed before administration of the first dose according to WHO toxicity criteria including an ocular examination for uveitis, ocular pressure

Table 5. Developmental anomalies and hemorrhages in fetuses of pregnant rats treated i.m. with 6.3 mg/kg Setarud during gestation.

	Number of animals	
	Control	Setarud
External hematoma	0	0
Hematoperitoneum	0	0
General edema of embryo	0	0
Hematoma in the body tissue	0	0
Hydronephrosis	1 (4%)	1.5 (10%)*
Hemopericardium	2 (8%)	3 (20%)*

* Statistically significant ($p < 0.05$)

Table 6. The absence of ossification centers in the skeletons of fetuses in pregnant rats treated with 6.3 mg/kg i.m. Setarud in the gestation period.

The absence of ossification centers in the skeletons of fetuses	Percent of animals	
	Control	Setarud
Breast bone	3.5	1.8
Kyoid bone	0	59.3*
Anterior limb bones		
2nd metacarpal bone	15.6	36.7*
3rd metacarpal bone	0	6.7*
4th metacarpal bone	12.5	26.7*
Posterior limb bones		
2nd metatarsal bone	15.6	30.0*
3rd metatarsal bone	0	26.7*
4th metatarsal bone	12.5	26.7*
Torso bones		
Ischial bone	0	0
Iliac bone	0	26.7*
Pubic bone	15.6	40.0*

* Statistically significant ($p < 0.05$)

measurement and an Electrocardiogram (ECG). For the first two days, patients were actively monitored for 4 hrs following drug administration for observed any possible adverse effects. To identify possible DLT manifestations, patients were visited and examined by a specialist physician every week. CD4 counts for all patients were recorded one and two months after the treatment period to investigate delayed complications.

Statistical analysis

The results from the experiments were expressed as the mean ± SE. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Parental necropsy findings, sex ratio of fetuses, and skeletal, and visceral anomalies were compared using Chi-squar test. Fisher exact test was used when needed. Non parametric tests of Kruskal-Wallis and Mann-Whitney were also carried out because of small numbers in groups. *P* values of <0.05 were considered statistically significant.

RESULTS

LD_{50} and acute toxicity

The LD_{50} for Setarud in male vs. female BALB/c mice were determined to be 1980 vs. 1860 and 1680 vs. 1680 mg/kg for i.m. and i.p. injections, respectively. In Wistar rats, the LD_{50} indices for Setarud were calculated to be 1830 and 1620 mg/kg i.m. and i.p., for male and 1260 and 1500 mg/kg, correspondingly. Both i.m. and i.p. injections of Setarud in doses close to LD_{50} were accompanied by expressed depression, narcosis and sleep in animals. In autopsy, plethora of most internal organs was marked.

Chronic toxicity; General state and behavior

Body weight of rats that received 6.3 mg/kg/day of Setarud significantly increased after 1 and 3 months of treatment ($p < 0.05$). Intramuscular

Table 7. The indices of postnatal development of young rats prenatally exposed to Setarud.

	Animal groups	
	Control	Setarud
Stillbirth (%)	0	5.9*
Postnatal mortality (%)	3.6	3.1
Body weight (g)		
At birth	6.6 ± 0.3	5.7 ± 0.3
7 days of life	15.0 ± 0.9	9.6 ± 0.5*
14 days of life	28.3 ± 1.1	18.8 ± 1.0*
21 days of life	35.0 ± 1.8	32.0 ± 0.2*

* Statistically significant ($p < 0.05$)

injection of Setarud at the dose levels of 2.1 and 6.3 mg/kg for 3 months had no adverse effects on general state and behavior of rats. The animals had smooth hair, they ate fodder willingly and their motor activity was unchanged. Prolonged injection of Setarud in the doses of 2.1 and 6.3 mg/kg/day into thigh muscles of animals did not show local irritating effects.

Hematological and biochemical parameters

Duriog chronic administration of Setarud to rats, statistically significant changes in the levels of erythrocytes, leukocytes, thrombocytes and hemoglobin were not observed. The blood chemistry results showed no significant change in levels of total protein, alkaline phosphatase activity, ALT, AST, LDH, total bilirubin, glucose, total cholesterol, triglycerides, urea and creatinine after 1 and 3 months treatment of rats (Table 3) with Setarud when compared with samples taken from the animals prior to the first injection and from controls. There were no statistically significant differences between female and male rats in above mentioned parameters ($p > 0.05$).

Histopathological examination

Macroscopic and microscopic examination of the major organs in rats treated with 2.1 and 6.3 mg/kg/day of Setarud for 3 months revealed no evidence of abnormality and pathological changes of the internal organs.

Embryotoxicity and teratogenicity

Body weight of pregnant female rats that received i.m. injection of Setarud at a dose of 6.3 mg/kg did not change significantly in comparison with the control group after 1, 2 and 3 gestational weeks. Total scores for evaluation of embryotoxicity during pregnancy, including number of live fetuses, implantation sites, yellow bodies, and embryo body weight were significantly lower in the Setarud group ($p < 0.05$) (Table 3). While compared to the control group, Pre-implantational deaths in the Setarud

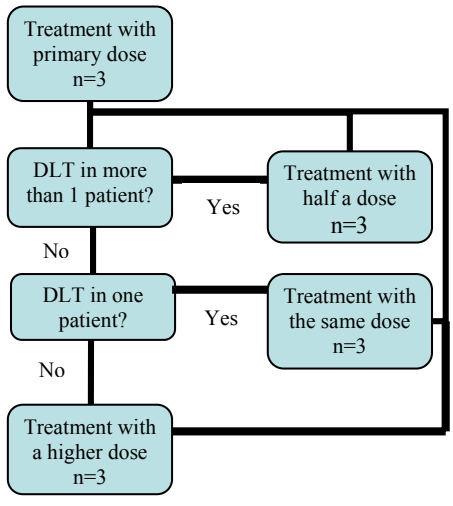

Figure 1. Study design for MTD determination of Setarud.

group were significantly higher, post-implantational deaths level were lower. Cranio-caudal size of the fetuses of pregnant rats in the Setarud group was not statistically different from that of control group (Table 4).

Macroscopic and micro-anatomic examinations of the fetuses that were exposed to Setarud in their prenatal period showed significantly higher incidence of hydronephrosis and hemopericardium than those of control group (Table 5). No developmental defects of the skeleton were detected. However, inhibition of ossification in the skeletons of the fetuses in the Setarud group was significantly higher than in control group (Table 6).

Incidence of stillbirth in the treatment group was higher than in the control group. Body weight of new-born rats of treatment group in the first month of their lives were lower than those of control group. Postnatal mortality did not differ significantly between two groups (Table 7). The development of new-born rats according to hair growth, appearance of incisors, opening of eyes, development of ears, vagina opening, and descent of testis and transitional periods of reflex maturation were largely comparable between two groups.

Patient characteristics

Totally, 12 HIV positive male patients were treated with escalating doses of intravenous Setarud. The median age and weight of patients were 34 years (range, 21 to 60 years) and 71.5 kg (range, 53 to 87 kg), respectively.

Doses and toxicities

In the first cohort, two patients experienced sweating and mild weight loss (< 5% of their body weight) which was improved without discontinuation of

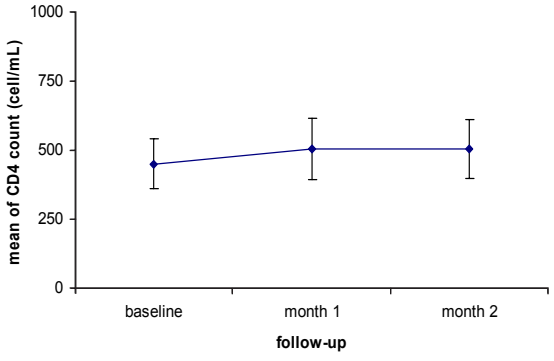

Figure 2. Mean ± confidence interval 95% CD4 count at baseline and follow-up periods

the drug. No other significant side effect or toxicity was observed in this group. There were also no side effects in second, third and fourth cohorts at doses of 4, 6.7 and 10 ml respectively.

Immune response

For evaluation of virologic and immunologic activity, we pooled the results across cohorts. Among 12 patients, no significant change in HIV RNA log10 copies/ml from baseline to month 1 and 2 was detected (P= 0.937 and P= 0.594, respectively). The median decrease in HIV RNA level was 0.08 log10 copies/ml after 2 months. Median increases of 12.5 and 23 CD4 cells/ml were observed from baseline to month 1 and 2 (P= 0.638 and P= 0.758, respectively) (Figure 2).

DISCUSSION

The results show that toxicity of Setarud did not differ significantly between i.m. and i.p. modes of injection. No significant sexual differences was identified in the sensitivity of the given animal species to the toxic effects of Setarud. Thus, it is concluded that Setarud is a low-toxic drug when injected i.m. or i.p. to BALB/c mice and Wistar rats.

There were no significant changes in animal's general state and behavior. Only the body weight increased significantly in rats treated with 6.3 mg/kg/day of Setarud for 3 months. No significant change occurred in hematological and biochemical parameters of the animals. The absence of significant changes in total protein, glucose, cholesterol and triglyceride levels revealed that long term administration of Setarud to rats did not produce any undesirable effects on protein, carbohydrate and lipid metabolisms. The liver enzymes activity and bilirubin levels did not show significant changes after administration of Setarud which suggested that Setarud did not exert any adverse reaction on liver. The absence of considerable changes in urea and creatinine levels made it possible to conclude the absence of the damaging effect of Setarud on the excretory function of the kidney in the experimental animals. According to the data of histopathological examinations, no toxic effects of Setarud on animals were observed.

However, the results of embryotoxicity and teratogenicity assay showed that the drug had the embryotoxic and teratogenic properties in rats at the dose level of 6.3 mg/kg. Our data are in agreement with previous findings that show embryotoxicity of Selenium in hamsters with a dose-response relationship (32). Thus Setarud is not recommended for administration during pregnancy.

In this Phase I clinical trial (without control group), all patients were treated with escalated doses of the drug which started out at 2 ml/day. The doses were easily tolerated up to level of 10 ml/day. None of the patients included in the study experienced any serious side effects with escalating dose of Setarud. Despite absence of adverse effects the study was terminated at 10 ml of a 125/4 ml.

The main objective in a Phase I clinical trial is to find MTD of the drug (33) with a higher probability of response and no or acceptable toxicity (34). In fact, the underlying assumption of all phases I clinical trials is that the dosage of a drug is related to probable toxic response (35). Since the dose established as the MTD will be passed for further testing in phase II clinical trials, accurate determination of the MTD is of grave importance (36). Perhaps, because phase I clinical trials are generally non-randomized, do not require large sample sizes, and are not hypothesis-driven, statistical considerations are largely ignored (35).

Conducting a phase I clinical trial on herbal extracts is not an essential part of the process to develop a new herbal drug. Traditional herbal remedies usually have been subject to extensive use by general population and all possible side effects are normally well known for these agents. However in order to gather clinical and para-clinical information for different concentrations of Setarud in a systematic approach with careful monitoring and scheduled recordings it was decided to conduct this study.

In this study the dose was not increased to maximum tolerated dose. To achieve this goal, it was necessary to expose participating subjects to extremely high levels of Setarud. As the study started with 10% of LD_{10}, the dose was increased 5 times in four successive steps, it was agreed that the dose range used in these subjects is wide enough to create an acceptable therapeutic window for effective use of the drug. Therefore there was no need to go beyond this level. Furthermore in the next stage a phase II clinical trial had been planned for this drug. This would have given us more accurate information of therapeutic effect of the drug.

Findings from this phase I clinical trial opens the way for the next phase of investigations. It can therefore be stated that Setarud is a safe drug and

can be recommended for phase II clinical trials. Considering the limitations of HAART resulted partly from extremely high degrees of mutations in the virus's antigenic structure, introduction of drugs such as Setarud which is believed to have immune system stimulating properties may be a significant contribution towards relieving the pain of patients with HIV/AIDS.

ACKNOWLEDGMENT

The authors would like to express their gratitude to Pars Roos Co. for supporting these research.

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