Original Paper

Identification and Evaluation of Potential Systemic Toxicity of Recombinant Human Interleukin-2 in Wistar Rats Based on Therapeutic Dosing Regimen

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ABSTRACT

Recombinant human Interleukin-2 (rhIL-2) continues to be in therapeutic application for the treatment of melanoma and renal cell carcinoma. A regimen of several, repeated dose, daily intravenous injections for 14 or 28 days duration has been shown to induce mortality prior to scheduled terminal sacrifice of the animals. The conventional study design not only manifests in pre-terminal mortalities but also minimizes the characterization of toxic profile of the molecule. In humans, each intravenous treatment exposure involves two five-day treatment cycles separated by 9 days of rest period.

The current 28-day study has been designed with three cycles of treatment and two rest periods, each treatment period comprising 5 days of consecutive treatment with 6 or 7 days of rest period. The study included three dose levels at 10, 20 and 40 times the human dose.

Treatment related clinical signs such as reduction in the spontaneous activity of animals, abdominal breathing and scruffy coat were noticed in a few animals treated at mid- or high-dose levels.

Treatment induced adverse changes were apparent in platelet counts and plasma activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT) at mid- or high-dose levels.

Histopathology revealed inflammatory cell infiltrates and/or its associated degenerative/regenerative changes in lung, liver and kidneys. The pharmacodynamic response, increase in white pulp was observed in the spleen, which was also evident in the total leukocyte count (WBC count) with a primary increase in the counts of lymphocytes and eosinophils. At the lowest dose level, which was 10 times the human dose, there were no manifestations of major adverse toxicity.

Key Words: Recombinant human interleukin-2; rhIL-2

Introduction

Interleukin-2 (IL-2) is a protein that occurs naturally in the body and plays an important role in activating the immune system. Recombinant Interleukin-2 (rhIL-2) is a recombinant version of IL-2, possesses the same properties as naturally occurring IL-2, and helps as an immunomodulator by stimulating T cell and B cell proliferation and differentiation. IL-2 mediated proliferation of nonspecific cytotoxic effector cells such as natural killer cells and lymphokine-activated killer cells, and the activation of monocytes and macrophages have been demonstrated. Recombinant human interleukin-2 (rhIL-2) continues to be employed in medical practice primarily for the treatment of metastatic melanoma and metastatic renal cell carcinoma, despite its dose-limiting toxicities.[1,2] Many non-clinical toxicology studies conducted by repeated daily intravenous injections revealed severe dose-limiting toxicities and/or pre-terminal mortalities prior to scheduled sacrifice, even at or close to human extrapolated doses. To determine the maximum tolerated dose, along with its targeted pharmacological effects, and to characterize manifestation of systemic toxicities, a sub-

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acute toxicity study has been designed by considering the human therapeutic dosing regimen.

Materials and Methods

Test Species and Husbandry: Healthy Wistar rats (42 males and 42 females) approximately 5-6 weeks old, obtained from the Laboratory Animal Research Services, Reliance Life Sciences Pvt. Ltd., were used as the test system. The rats were maintained in an environment-controlled room, with temperature maintained at 22 ± 3°C and relative humidity of 50-65%. In the experimental room, 12 hours of artificial fluorescent lighting and 12 hours of darkness were maintained. The animals were housed in solid floor polypropylene rat cages with rice husk as a bedding material. The animals were provided with *ad libitum* laboratory rat feed and charcoal filtered, UV sterilized water.

Recombinant Human Interleukin-2 (rhIL-2), purified from *Escherichia coli* through recombinant technology, manufactured by Reliance Life Sciences Pvt. Ltd. with biological potency of 17.9 X 10^6 IU/mg was used for the study.

Study Design: The animals were randomly allocated to 7 groups. Each group comprised 6 male and 6 female rats. At the start of the treatment, body weight variation among the animals was within the ± 20% of mean body weight range.

Three dose levels, viz., 1.1, 2.2 and 4.4 (low, mid and high dose) mg/kg were selected based on available literature. The selected dose levels in the rats were 10, 20 and 40 times higher than the intended human intravenous daily dosage. In addition, a market product (as a comparator) was also tested at 1.1 mg/kg. The 28-day treatment period comprised three cycles of treatment with two rest periods. The animals were injected intravenously on days 1-5, 13-17 and 24-28 of experimentation. Recovery group animals were kept under observation for a further period of 14 days to find out reversibility, and persistence or delayed occurrence of toxic effects, if any. The control group animals were given the vehicle only. The study was conducted as per international guidelines/standards.[3-5]

Results

In-life Phase Parameters: All animals were observed carefully for any signs of illness or reaction towards treatment at least once a day after dose administration. All visible signs of reaction to the treatment were recorded. Detailed clinical examination was performed at approximately weekly intervals thereafter. Body weight and food consumption were recorded individually for all animals on days 1, 6, 13, 18, 24, 28, 29, 35 and 42.

Clinical Pathology Observations: After 4 weeks of treatment period, all animals from each group were fasted overnight (water allowed). On test days 29 and 30, for males and females respectively, blood samples were drawn from retro-orbital plexus under light ether anaesthesia. For clinical chemistry, blood was collected using lithium heparin as anti-coagulant, and the resultant plasma used for analysis. Haematology was done using potassium EDTA stabilized blood.

The following haematology parameters were evaluated using haematology analyzer, Medonic CA 620: total leukocyte count (WBC), erythrocyte count (RBC), haemoglobin (HGB), haematocrit (HCT), platelet count (PLT) and erythrocyte indices. Blood smears made from fresh samples were stained with Leishman's stain and subjected to differential leucocyte count (DLC), manually. The plasma profiles anlaysed by Dade Behring clinical chemistry analyzer included albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), blood urea nitrogen, calcium, total cholesterol, creatinine, glucose, phosphorus, total bilirubin, total protein, triglycerides, sodium, potassium and chloride. Microscopic and qualitative urinalysis was conducted on all animals at the end of the treatment and recovery periods. Urinalysis included the study of colour, clarity, volume, specific gravity, glucose, bilirubin, ketones, occult blood, pH, protein and urobilinogen. Urine sediment was observed under light microscope for pus cells, RBCs, epithelial cells, casts and crystals.

Histopathology: On test day 29, all animals from the main group were humanely euthanised by carbon dioxide asphyxiation. Each animal was subjected to a full, detailed gross necropsy which included careful examination of the external surface of the body, all orifices, the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, ovaries, thymus, spleen, brain and heart of all the animals were trimmed of adherent tissue, and their wet weight taken before fixing.

The organs/tissues selected for microscopic examination included adrenals, aorta, brain (including cerebrum, cerebellum, and medulla /pons), caecum, colon, duodenum, epididymides, oesophagus, eyes, female mammary glands, femur, gonads, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, vagina, uterus, pancreas, urinary bladder, peripheral nerve (sciatic nerve), pituitary, prostate, trachea, salivary glands, seminal vesicles, skin, skeletal muscle, spinal cord (cervical, mid thoracic and lumbar regions), spleen, sternum, stomach, thymus, thyroid/parathyroid, site of injection, femur with marrow, and all gross lesions. Histopathological examination of haematoxylin-eosin stained paraffin sections were performed on representative samples of all tissues listed above, of all animals in the control and high dose groups. In addition, liver, lung, kidneys, spleen and injection sites of mid, low, comparator low-dose groups, control recovery and high dose recovery animals were examined microscopically for treatment related changes at the high dose level.

Evaluation of Data: Statistical analysis was done using validated statistical software. All the parameters characterized by continuous data such as body weight, food consumption, relative organ weight, haematology, and clinical chemistry data were subjected to statistical analysis using Bartlett's, ANOVA and t-test. Significance was calculated at 5% level.

The rats treated at 2.2 and 4.4 mg/kg dose levels revealed predominant treatment related responses in terms of clinical signs, haematology, plasma chemistry and histopathological changes. These treatment related changes included both pharmacological effects and adverse effects. During days 7-9 and 27-30 of experimentation, a few animals belonging to mid-dose and high-dose groups exhibited clinical signs such as transient reduction in spontaneous activity and/or abdominal breathing. In addition, two females, one each belonging to mid- and high-dose groups developed a scruffy coat.

Haematological parameters revealed a significant increase in the counts of total WBC in all the treatment group males, whereas females showed dose-dependent increasing trend with statistical significance at high-dose level (**Figs 1 and 2**). The total leucocyte increase was primarily associated with a higher increase in the counts of lymphocytes and eosinophils; however, neutrophil and monocyte counts also increased marginally in treated animals. Further, the rat blood analysis revealed a decrease in the concentration of platelet count, predominantly in mid- and high-dose animals at the end of the treatment period (**Table 1**).

Plasma chemistry revealed increase in activities of ALT, AST and GGT, and total bilirubin concentration, particularly at 2.2 and 4.4 mg/kg dose levels (**Tables 2 and 3**). Reversibility to normalcy, or a trend towards normalcy was observed at the end of the recovery period.

Internal gross examination revealed splenomegaly in all the treatment group animals at terminal and recovery sacrifice. Histopathological changes were primarily related to liver, lungs, kidneys and spleen. The microscopic changes observed in the liver included hepatocellular degeneration, perivascular and periportal inflammatory cell infiltration, fibrotic bridges across hepatic parenchyma (one high-dose animal), and bile duct proliferation. Lungs showed inflammatory responses such as perivascular and periportal inflammatory cell infiltration, Clara cell activation and peneumonitis. Microscopy of kidneys revealed perivascular MNC infiltration, tubular degeneration, tubular regeneration and tubular cystic dilatation. Spleen showed capsular thickening and hypertrophy of white pulp (**Table 4**). At the injection site, perivascular MNC and PMN infiltration, and perivascular fibrotic proliferation were observed in animals belonging to control, and different treatment group animals.

Discussion

The therapeutic application of IL-2 depends on tumour regression through immunomodulation. Its adverse effects are also in relation to its primary therapeutic effect of increasing the various kinds of cells such as T cells, B cells and nonspecific cytolytic effector cells. In the current study, pharmacological effects and adverse findings were apparent both biochemically and histopathologically. The adverse changes noticed were concluded to be secondary changes to primary exaggerated pharmacological response.

The pharmacological findings were apparent in all the groups of both the sexes, which were very much dose-dependent. Haematologically, the rats exhibited leukocytosis, lymphocytosis and eosinophilia. Corroborative findings were evident in the white pulp of spleen. This increase in the white pulp of spleen was also reflected as an increase in the size of spleen in all the treated groups. The increase is primarily due to the anticipated

Table 1 Platelet-Group Mean Values
Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day

Parameter/Group		Control	Low	Mid	High	Comparator
Sex: Male						
PLT (10^3/mm^3)	Mean	736.0	685.2	645.8	438.5*	546.5*
	SD	51.6	80.2	158.7	89.8	73.1
Sex: Female						
PLT (10^3/mm^3)	Mean	623.7	580.5	518.7	476.3*	530.8
	SD	116.6	106.4	104.9	63.8	57.4

* = Significant ($p \leq 0.05$)

Table 2 Plasma Chemistry-Group Mean Values: Males
Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day

Parameter/Group		Control	Low	Mid	High	Comparator
ALT (U/L)	Mean	52.83	55.50	50.50	74.17*	52.50
	SD	18.10	4.18	6.95	18.8	6.09
AST (U/L)	Mean	77.8	94.3	86.3	138.3*	88.00
	SD	16.2	14.6	13.0	31.4	8.85
GGT (U/L)	Mean	2.0	1.83	3.33	6.17*	5.33*
	SD	1.27	2.48	1.86	3.87	1.03
TBIL (mg/dL)	Mean	0.15	0.15	0.18	0.30*	0.20*
	SD	0.03	0.02	0.02	0.09	0.04

* = Significant ($p \leq 0.05$)

Table 3 Plasma Chemistry-Group Mean Values: Females
Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day

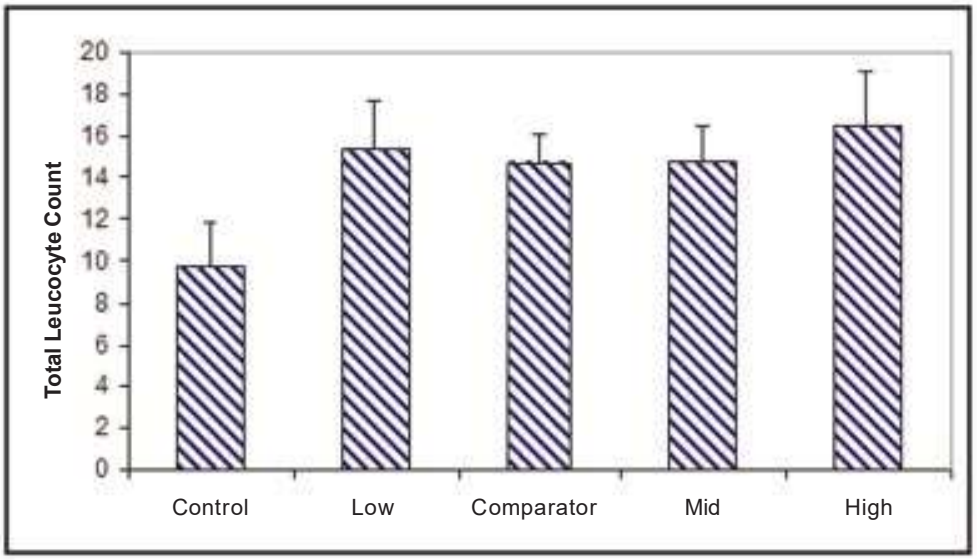
Parameter/Group		Control	Low	Mid	High	Comparator
ALT (U/L)	Mean	43.2	47.3	50.0	55.50*	38.67
	SD	9.0	6.8	2.1	4.85	1.37
AST (U/L)	Mean	65.3	81.8	90.5*	106.0*	78.83*
	SD	11.6	5.6	13.3	24.0	8.42
GGT (U/L)	Mean	3.0	4.5	3.50	4.83*	3.00
	SD	0.6	1.5	0.55	1.47	1.41
TBIL (mg/dL)	Mean	0.14	0.13	0.17	0.23*	0.29*
	SD	0.02	0.02	0.07	0.04	0.04

* = Significant ($p \leq 0.05$)

Table 4 Gross and Histopathological Findings - Summary by Group
Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day

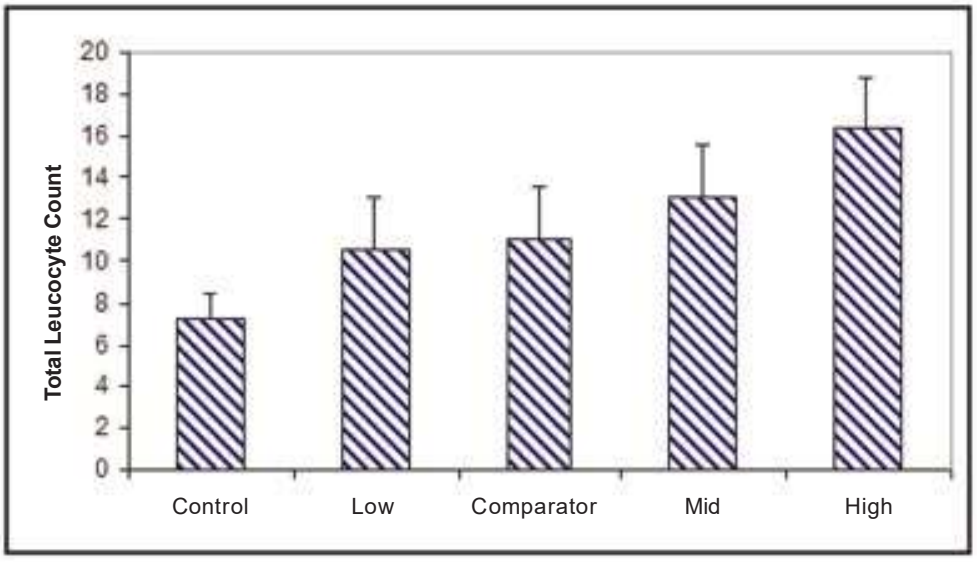
Groups	Low		Mid		High		High Recovery		Comparator	
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Lungs										
Perivascular inflammatory cell infiltration	1 (c)	1 (c)	1 (d)	3 (d)	6 (d)	6 (d)	5 (b)	6 (a)	6 (b)	3 (b)
Clara cell activation	6 (b)	6 (b)	6 (c)	6 (c)	6 (c)	6 (c)	0	0	4 (a)	6 (b)
Pneumonitis	6 (c)	6 (c)	6 (c)	6 (c)	6 (c)	6 (c)	0	0	6 (a)	6 (b)
Liver										
Hepatocellular degeneration	3 (b)	6 (b)	6 (c)	6 (c)	6 (c)	6 (c)	4 (b)	3 (b)	6 (b)	6 (b)
Perivascular & periportal inflammatory cell infiltration	6 (b)	6 (b)	6 (c)	6 (c)	6 (c)	6 (c)	6 (b)	6 (b)	6 (b)	6 (b)
Fibrotic bridges across hepatic parenchyma	0	0	0	0	1 (c)	0	0	0	0	0
Bile duct proliferation	6 (c)	6 (c)	6 (c)	6 (c)	6 (c)	6 (c)	6 (b)	6 (b)	6 (c)	6 (c)
Kidneys										
Perivascular inflammatory cell infiltration	1 (a)	0	0	0	4 (c)	4 (c)	0	0	0	0
Tubular degeneration	1 (a)	0	0	0	2 (c)	3 (c)	0	0	0	0
Tubular regeneration	1 (a)	0	1 (b)	2 (b)	4 (c)	4 (c)	3 (a)	4 (a)	4 (a)	3 (a)
Tubular cystic dilatation	0	0	0	2 (b)	2 (b)	1 (b)	1 (a)	3 (a)	0	2 (b)
Spleen										
Splenic capsular thickening	6 (a)	6 (a)	6 (b)	6 (b)	5 (b)	6 (b)	4 (b)	6 (b)	6 (a)	6 (a)
Hypertrophy of the white pulp	6 (b)	6 (b)	6 (c)	6 (c)	6 (d)	6 (d)	6 (b)	6 (b)	6 (b)	6 (b)

M = Male, F = Female, (a) - Minimal, (b) - Mild, (c) - Moderate, (d) - Severe
Numerals in the table represent the number of animals showing particular lesions



* = Significant ($p \leq 0.05$)

Fig. 1 Total Leucocyte Count ($10^3/mm^3$): Males (Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day)



* = Significant ($p \leq 0.05$)

Fig. 2 Total Leucocyte Count ($10^3/mm^3$): Females (Dose: G1 - Control; Low - 1.1; Mid - 2.2; High - 4.4; Comparator - 1.1 mg/kg bwt/day)

pharmacological effect associated with the treatment with rhIL-2. In all the treated animals, splenomegaly was obvious both grossly and histopathologically. Concurrent to the haematology findings, inflammatory cell infiltrates were observed primarily in the liver, lungs and kidneys.

The adversity associated with exaggerated pharmacological findings is a common finding of biosimilars after repeated exposure and/or exposure above the therapeutic limit. Secondary effects to exaggerated pharmacodynamic actions are also not uncommon. The degenerative/regenerative/inflammatory responses observed in liver, kidneys and/or lung were primarily owing to increased inflammatory cell infiltrates and the subsequent mechanism associated with it, which is considered to be a secondary systemic adverse effect associated with the anticipated immunomodulating effect of rhIL-2. Corroborative changes were also noticed in the activities of ALT, AST and GGT, and in the total bilirubin concentration. Both the severity and the incidence of all the lesions were much less in the recovery animals.

Decrease in the concentration of platelet count was apparent in treated animals, considered to be pathological changes associated with repeated IL-2 administration. There have been studies relating to platelet-endothelium interactions after IL-2 exposure, in which it has been reported that the effects on the endothelium decrease platelet disaggregation after repeated IL-2 treatment, which might end up in a microvascular thrombus formation and contribute to systemic toxicity.[6] Immunotherapy using IL-2 has also revealed thrombocytopenia in a number of patients. This has been hypothesized to be due to increased destruction of platelets by the reticuloendothelial system, leading to thrombocytopenia.[7]

Mediators of blood cells such as lymphokine-activated killer cells, NK cells and neutrophils were found to be associated with rhIL-2 toxicity. The vascular leakage syndrome and lymphokine-activated killer cells are the two important mechanisms associated with the rhIL-2 mediated toxicity. Treatment with rhIL-2 causes increase in the concentration of TNF level in a dose dependent manner, which in turn induces the increased expression of endothelial leukocyte adhesion molecules, and causes vascular endothelial permeability and injury to organs. The role of rhIL-2 induced lymphokine-activated killer cells is also a dose-dependent mechanism. IL-2 activated lymphocytes, in addition to tumour cell lysis, have shown enhanced adhesion to normal vascular endothelial cells, and cause toxicity by the lysis of cells.[8-11]

At 1.1 mg/kg dose level, tested products did not reveal any evident adverse changes in the behaviour, growth, haematology and clinical chemistry parameters. A low level of inflammatory cell infiltrates, particularly in the lung and liver did not reveal any serious adverse changes, at this dose level. Pharmacological effects of rhIL-2 were also predominant at 1.1 mg/kg and considered to be well tolerated. The market product (as a comparator) tested at 1.1 mg/kg revealed a similar pharmacological response and concomitant inflammatory cell infiltrates in the lungs and the liver.

Pre-terminal mortality or termination of the study prior to scheduled sacrifice not only invalidates a study, but also provides limited information on the systemic toxic profile of a candidate drug/test item. Varying the study design/dosing regimen several times can help in understanding the toxic manifestations, and to derive a safe dose level. Understanding systemic exposure/bioavailability and its sustained pharmacological effects, is very essential in the design of non-clinical toxicology studies.

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