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Toxicology and Applied Pharmacology Preclinical Safety Evaluation of SPRC, a Novel H_2S Donor, in Rats

Xianliang He[1*], Wei Qi[1*], Rinkiko Suguro[1#], Sei Suguro[2], Chen Fan[1], Yizhun Zhu[1#]

[1]Laboratory for Drug Discovery from Natural Resource, Research and Development Center, Macau University of Science and Technology, Macau SAR, China; [2]Faculty of Medicine, School of Pharmacy, The Chinese University of Hong Kong, Hong Kong SAR, China

Correspondence to: Rinkiko Suguro, rsuguro@must.edu.mo; Yizhun Zhu, yzzhu@must.edu.mo
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ABSTRACT

Hydrogen sulfide (H_2S) is a crucial signaling molecule involved in regulating inflammation, oxidative stress, and metabolism. S-propargyl-cysteine (SPRC) modulates endogenous H_2S production pathways by influencing relevant enzymes, presenting a potential therapeutic approach for conditions associated with abnormal sulfide levels. This study investigated the safety and pharmacokinetic properties of SPRC in a Good Laboratory Practice 28-day oral toxicity study in rats, including safety pharmacology and gene toxicity assessments. SPRC exhibited favorable pharmacokinetic characteristics and safety margins, with a No Observed Adverse Effect Level (NOAEL) of 37.5 mg/kg, translating to an estimated 8.5-fold therapeutic window. These initial toxicology findings, coupled with SPRC's pharmacological effects on the sulfide signaling axis, support its progression to first-in-human clinical trials for further benefit risk evaluation. The promising safety and pharmacokinetic profile of SPRC underscores its therapeutic potential and justifies additional clinical investigation for disorders linked to aberrant sulfide signaling. To comprehensively assess the therapeutic utility of SPRC, further preclinical safety evaluations and early phase clinical studies are warranted. These investigations will provide insights into SPRC's potential to address unmet medical needs in various disease areas related to disrupted sulfur metabolism and hydrogen sulfide biology.

1. INTRODUCTION

S-propargyl-cysteine (SPRC) is an emerging investigational agent with therapeutic potential across

*These authors contributed equally.

#Corresponding author.

various disease areas. Structurally, SPRC contains a propargyl moiety conjugated to the sulfur atom of cysteine. It primarily functions through interaction with cystathionine β-synthase (CBS), a key enzyme involved in endogenous hydrogen sulfide (H_2S) production [1, 2]. H_2S has surfaced as a crucial signaling molecule with diverse physiological roles, including vasodilation, inflammation, and redox modulation [3-5]. This report aims to develop SPRC as a potential therapeutic agent. It is essential to comprehensively evaluate its toxicokinetics, safety, and therapeutic potential in preclinical models. The lack of systematic toxicokinetic and safety data on SPRC hinders its translation into clinical studies. Moreover, understanding the therapeutic potential of SPRC in various disease models is crucial to identify the most promising indications for further development. The rationale behind this study is to bridge the gap in knowledge of translational medicine regarding the toxicokinetics, safety, and therapeutic potential of SPRC in rats. By conducting a thorough preclinical evaluation, this study aims to provide a solid foundation for the development of SPRC as a novel H_2S-modulating therapeutic agent. The growing interest in harnessing the therapeutic potential of H_2S has led to the development of H_2S-releasing drugs, such as SPRC. Here, we provide an overview of the current understanding of SPRC in a preclinical setting, its therapeutic potential, and the challenges and future directions in its development as a drug candidate.

The discovery of H_2S as a physiologically relevant signaling molecule has sparked interest in its therapeutic applications. SPRC, an analog of the nutraceutical S-allyl cysteine (SAC) found in garlic extracts, has emerged as a promising candidate for modulating endogenous H_2S production [6, 7]. Early studies have demonstrated the cardiovascular and antioxidant effects of cysteine derivatives like SPRC, paving the way for further exploration of their therapeutic potential [8, 9].

Recent studies have shed light on the multifaceted mechanisms of action of SPRC. By interacting with cystathionine β-synthase (CBS), a key enzyme in endogenous H_2S production, SPRC can modulate various pathways related to oxidative stress and immune responses [10, 11]. Preclinical investigations have revealed diverse pharmacological applications of SPRC, including tissue protection from ischemic injury, cardiovascular effects in heart failure models, proangiogenic capacity, and amelioration of arthritic changes [8, 9, 12-18]. Importantly, SPRC exhibits favorable pharmacokinetic properties of good oral bioavailability, and systemic distribution including blood, liver and kidney, and urinary excretion as the primary clearance pathway [19, 20]. The prolonged retention facilitates sustained therapeutic action [21]. We have undertaken an array of preclinical studies to systematically assess toxicity profiles, identify primary target organs, characterize reversibility of damage, and project human equivalent doses and therapeutic indices, thereby enabling first-in-human trials and providing preliminary clinical guidance on dosing monitoring biomarkers, and safety margins. The focus includes plasma exposure correlations with pharmacological activity and adverse effects to nominate a valid no-observed adverse effect level (NOAEL).

Despite the promising preclinical findings, several challenges and knowledge gaps remain in the development of SPRC as a therapeutic agent. The complex nature of H_2S signaling and its context-dependent effects require further elucidation to fully understand the therapeutic potential and limitations of SPRC. Additionally, the safety and pharmacokinetic profiles of SPRC need to be thoroughly characterized to enable its translation into clinical trials.

To address the current challenges and knowledge gaps, future research should focus on systematically assessing the toxicity profiles, identifying primary target organs, characterizing the reversibility of damage, and projecting human equivalent doses and therapeutic indices of SPRC. The integration of efficacy signals from disease models with safety and pharmacokinetic data will be crucial in supporting the clinical evaluation of SPRC as a prospective drug candidate harnessing hydrogen sulfide biology [5, 18]. Furthermore, exploring the potential of SPRC in combination with other therapeutic agents and investigating its efficacy in a broader range of disease models will help unlock its full therapeutic potential.

2. MATERIAL AND METHODS

2.1. Animals

Healthy male and female Sprague-Dawley (SD) rats aged 7 - 8 weeks (weight: 130.2 - 157.7 g) were

procured for the studies from Shanghai Xipu-Bikai Experimental Animal Co., Ltd. Experimental Animal Quality Certificate No.: 2008001611882, Serial No.: 0048872; Experimental Animal Production License No.: SCXK (Shanghai) 2008-0016, issued by Shanghai Science and Technology Commission; Experimental Animal Use License No.: SYXK (Shanghai) 2007-0003, issued by Shanghai Science and Technology Commission. The rats were randomized into groups (Table 1) for experiments and were housed at 22.0˚C - 23.1˚C and 55.1% - 62.1% humidity with a 12-hour light/dark cycle in polypropylene cages, allowing free access to standard rodent chow and water, except where noted. All rats were acclimatized for 3 days before initiation of any procedures.

Animal care and housing adhered to Good Laboratory Practice (GLP) guidelines throughout with Institutional Animal Ethics Committee approval. All animal procedures were conducted in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at Drug Safety Evaluation Center of Second Military Medical University. The study protocol was reviewed and approved by the IACUC prior to the initiation of the experiments. At the end of the study, animals were anesthetized using carbon dioxide and euthanized by carbon dioxide (CO_2) inhalation followed by cervical dislocation as a secondary method to confirm death. CO_2 will be delivered at a flow rate of 30% - 70% of the chamber volume per minute, as recommended by the Chinese Veterinary Medical Association (CVMA) Guidelines for the Euthanasia of Animals. The mice and rats will be kept in the CO_2 chamber until they are unconscious and have stopped breathing. Cervical dislocation will be performed immediately after CO_2 exposure to ensure death. Dogs were euthanized by intravenous injection of pentobarbital sodium (150 mg/kg) at the end of the study. The euthanasia procedure will be carried out by trained personnel in a dedicated euthanasia area.

2.2. Test Article

The tissue distribution of [^{35}S] SPRC (>99% purity)-derived radioactivity was evaluated in SD rats after an oral administration of 75 mg/kg [^{35}S] SPRC solution (25 μCi/mL, 10 g/L). SPRC-derived radioactivity and pharmacokinetic parameters measured using a liquid scintillation counter and mixed-mode reversed-phase and cation-exchange HPLC-MS/MS method have been respectively described previously [19, 20].

Propargyl-Cysteine (SPRC), CAS [3262-64-4], MW 159.21 with confirmed structural purity >99% was obtained from 2Y-Chem Ltd., China (Figure 1) and suitably formulated. S-butylcysteine served as the internal standard for bioanalysis.

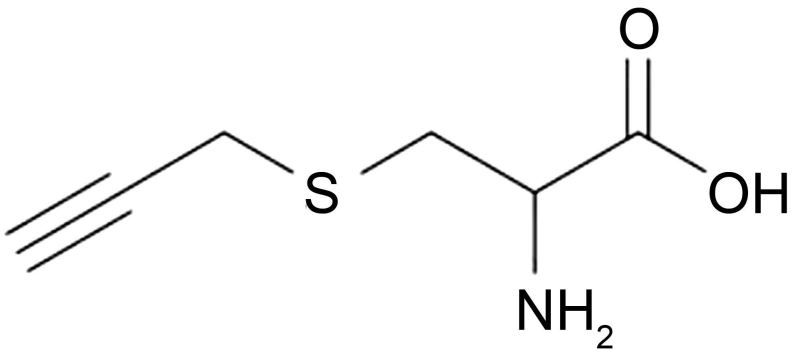


Figure 1. Structure of SPRC, CAS [3262-64-4], MW 159.21.

2.3. Treatment Groups

The 28-day toxicity study comprised daily oral gavage administration of SPRC at 0, 37.5, 75, or 150 mg/kg with appropriate group allocation and washout periods (n = 30/sex/groupx4) (Table 1).

Table 1. Number of animals in each group.

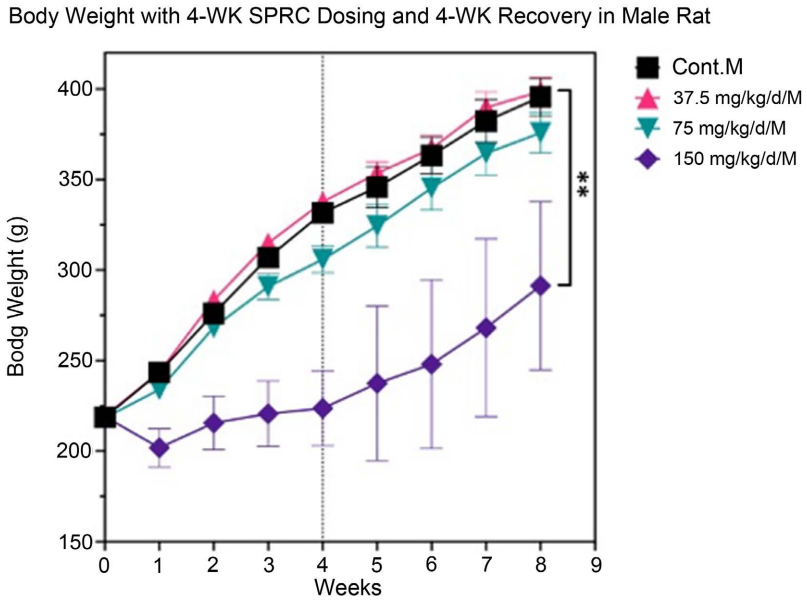
	Male	Female
Group	n	n
Control	15	15
37.5 mg/kg	15	15
75 mg/kg	15	15
150 mg/kg	15	15
Total: 120	60	60

2.4. Toxicity Assessments

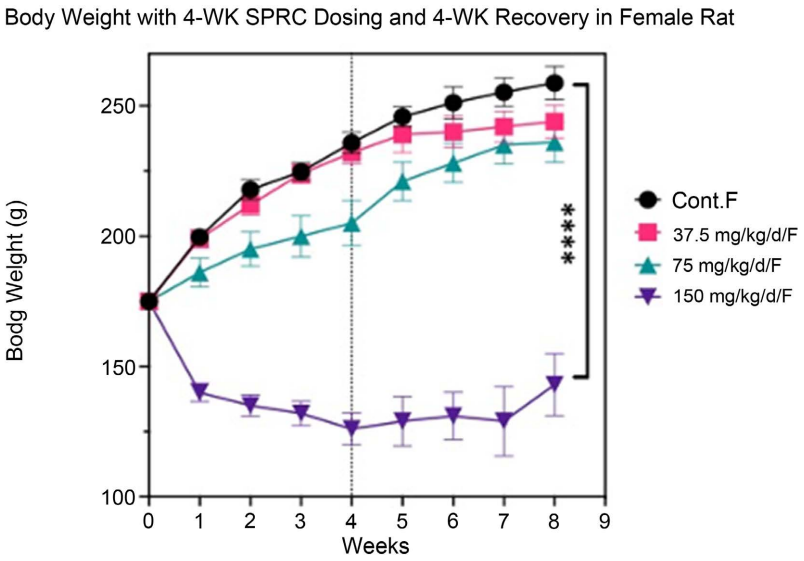
The 28-day toxicity assessment of SPRC incorporated a 28-day recovery phase aligned with the regulatory guidance. Key endpoints evaluated included, but not limited to, clinical signs, body weight, food consumption, clinical chemistry, gross pathology, and histopathology. SPRC doses were adjusted weekly based on individual rat weights over the 28-day dosing and recovery terms. All animal experiments complied with national and institutional animal use regulatory policies.

2.5. Safety Pharmacology Study

ICR mice were also procured for the study from Shanghai Xipu-Bikai Experimental Animal Co., Ltd., SPF grade, 6 - 7 weeks old, 18.4 - 21.4 g of body weight. 80 mice, half male and half female: 40 mice half male and half female for spontaneous activity test; other 40 mice half male and half female for pole climbing ability test. And 24 Beagle dog, ordinary grade, half male and half female, 7 - 9 months of age, 9.00 - 10.50 kg of body weight were used for the observation of the effects on the cardiovascular and respiratory system (Figure 2).



(A)



(B)

Figure 2. The body weight changes in (A) male and (B) female rats over a 28-day oral administration of SPRC at doses of 37.5 mg/kg, 75 mg/kg, and 150 mg/kg, followed by a 28-day recovery period. In the male group (A), the black solid square represents the control group, the pink solid upward triangle represents the 37.5 mg/kg group, the green solid downward triangle represents the 75 mg/kg group, and the purple solid diamond represents the 150 mg/kg group. In the female group (B), the black solid circle represents the control group, the pink solid square represents the 37.5 mg/kg group, the green solid upward triangle represents the 75 mg/kg group, and the blue solid downward triangle represents the 150 mg/kg group. Statistical analysis was performed using one-way ANOVA with multiple comparisons. Data are presented as mean ± SEM (n = 10 for the 28-day dosing period, and n = 5 for the 28-day recovery period). Significance is denoted by ** ($p < 0.01$) and ** ($p < 0.0001$).**

The mice were administered with single i.g. SPRC 50, 100, and 200 mg/kg and blank control group were used to observe the effects of the test substance on the spontaneous activity and climbing ability of mice with SPRC 50, 100, 200 mg/kg and treatment and blank as control. The doses were determined based on the rate Pk profile conversion between animals [22]. Mice were observed for spontaneous activity test and pole climbing ability test for any signs of toxicity or adverse effects.

In the beagle dog study, animals were procured for the studies from Shanghai Xingang Experimental Animal Farm. Experimental Animal Quality Certificate No.: 0073776, provided by Shanghai Xingang Experimental Animal Farm; Experimental Animal Production License No.: SCXK (Shanghai) 2007-0009, issued by Shanghai Science and Technology Commission; Experimental Animal Use License No.: SYXK (Shanghai) 2007-0003, issued by Shanghai Science and Technology Commission. Animals were administered with SPRC at doses of via i.g. Control animals received the same volume of solvent saline (2ml/kg). Main experimental steps: Beagle dogs were anesthetized by intravenous injection of 3% pentobarbital sodium saline solution at 30 mg/kg, fixed in a supine position, and cannulated in the femoral artery. Changes in systolic blood pressure, diastolic blood pressure, and mean arterial pressure were observed through a pressure transducer; the respiratory rate, amplitude, and rhythm of the animals were recorded through a tension transducer; and the standard limb lead II ECG of Beagle dogs was recorded through ECG leads. Dogs were observed after dosing at 5 min, 10 min, 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, and 180 min timepoints for any signs of toxicity or adverse effects. Cardiovascular parameters, including heart rate, blood pressure, and electrocardiogram (ECG), The above parameters were automatically monitored,

sampled and processed by the BL-420E+ biological function experimental system.

The experiments in Beagle dogs was divided into 4 groups: 4 mg/kg, 8 mg/kg and 16 mg/kg and blank control group (the doses were determined based on Pk profile conversion between animals and dog PK profile [21, 22]), respectively. There are 24 Beagle dogs, 6 in each group, half male and half female. Observe the effect of the test substance on the dog's systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, heart rhythm, ECG P wave voltage, T wave voltage, R wave voltage, QRS time, PR interval, QT interval, ST segment, and respiratory rate, rhythm and amplitude effects.

The genotoxicity testing battery was conducted to assess the mutagenic, micronucleogenic, and chromosome-damaging potential of SPRC. The three assays were: 1) Salmonella typhimurium reverse mutation assay (Ames test): Used S. typhimurium strains TA97, TA98, TA100, TA102, and TA1535 with tested doses: 5000, 3000, 300, 30, 3, and 0.3 μg/dish with and without metabolic activation (S9). 2) Mouse bone marrow micronucleus test: ICR mice administered SPRC orally at doses of 429.25, 858.5, and 1717.0 mg/kg. The positive control was intraperitoneal injection of phosphoramide (40 mg/kg). The bone marrow smears taken 24 and 48 hours after administration and the incidence of micronuclei in polychromatic erythrocytes was counted. 3) Chromosome aberration test in CHO cells: The tested doses were chosen at 750, 1500, and 3000 μg/ml (no obvious cytotoxicity observed), included solvent control and positive control groups. Cells collected and sliced 24 hours after administration. The chromosomes of 200 metaphase cells per dose group (100 for positive control) were observed and chromosome aberration rate was calculated accordingly.

3. STATISTICAL ANALYSIS

Body weight, food consumption, clinical chemistry, hematology, and anatomical pathology data were analyzed by one-way or two-way ANOVA followed by appropriate post-hoc tests for differences between control and treatment groups. Figures were generated and data was expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) using GraphPad Prism 10.0.2. Differences were considered statistically significant at $P < 0.05$. In complement, due to the significant difference in absolute values between males and females, pooling the data together may lead to statistical issues, as the significance of the results might be obscured. To avoid this problem, we analyzed the data separately for each sex to ensure that any significant differences are properly identified and interpreted.

4. HISTOPATHOLOGICAL ASSESSMENT

4.1. Examinations

All collected tissues and organs from animals were examined through histopathology to identify treatment-related lesions visually and based on organ weight and microscopy findings. Any observed toxicologic effects were further investigated for causality. Additionally, microscopic examination was conducted on lesions from all groups irrespective of attribution. Moribund or early deaths underwent rapid necropsy with visual organ inspection and histopathology.

4.2. Methods

Fixed organs were processed with graded ethanol dehydration, paraffin infiltration, and embedding using standard histology procedures. Paraffin blocks were sectioned at 3 μm thickness using a Leica RM2135 rotary microtome. The tissue sections were stained with hematoxylin and eosin (H&E) before microscopic evaluation. Slides were examined by light microscopy under 100× magnification using an Olympus BX51 system to assess degrees of pathological changes by an experienced veterinary pathologist.

5. RESULTS

Fixed organs were processed with graded ethanol dehydration, paraffin infiltration, and embedding using standard histology procedures. Paraffin blocks were sectioned at 3 μm thickness using a Leica RM2135

rotary microtome. The tissue sections were stained with hematoxylin and eosin (H&E) before microscopic evaluation. Slides were examined by light microscopy under 100× magnification using an Olympus BX51 system to assess degrees of pathological changes by an experienced veterinary pathologist.

In clinical chemistry, hepatic effects (increased ALT and bilirubin) and other changes indicative of nutritional deficiency which trended toward recovery (reference the Data in Brief article). Interestingly, in a radiolabeling PK study, significant liver weight loss showed dose dependent manner, where female appears more sensitive to SPRC (Figure 3) in liver weight and proportion to body weight loss. 75 mg/kg and 150 mg/kg rats displayed reduced absolute organ weights marginally in the heart, spleen, lungs, kidneys, and brain compared to control (Figure 3).

Figure 3. The absolute weight of the main organs in (A) male and (B) female rats after a 28-day oral administration of SPRC at various doses: saline control (green bar), 37.5 mg/kg (blue bar), 75 mg/kg (yellow bar), and 150 mg/kg (red bar). Statistical analysis was performed using a 2-way ANOVA with multiple comparisons. Data are presented as mean ± SEM (n = 10). Significance is denoted by ** ($p < 0.0001$).**

In radiolabeling [^{35}S] SPRC pharmacokinetic study, after oral administration (25 μCi/mg, 75 mg/kg) of SPRC, the main organs distribution of [^{35}S]-SPRC derived radioactivity was normalized by the corresponding time points of plasma radioactivity, the organs distribution increased in a time-dependent manner and passive distribution concentration gradient, except kidney. Kidney accumulation of [^{35}S] SPRC occurred significantly with time dependent manner compared to normalized plasma concentration, where the concentration shown higher than that of plasma (Figure 4), indicated anactive transport dynamic. In contrast, [^{35}S] SPRC of the heart showed a decreasing trend relative to plasma concentration over the first 6 hours, and the other main tissues showed a slightly increased pattern over the first 6 hours.

Table 2 presents the liver function parameters in male and female rats treated with the three doses of SPRC (37.5, 70, and 150 mg/kg/day) for 28 days, followed by a 28-day recovery period. The parameters include Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Total Protein (TP), Albumin (ALB), Total Bilirubin (TBIL), Alkaline Phosphatase (ALP), Blood Urea Nitrogen (BUN), Creatinine (CRE),

Total Cholesterol (TCH), Triglycerides (TG), Creatine Kinase (CK), and Lactate Dehydrogenase (LDH). Notable findings include significantly increased ALT levels in males at 150 mg/kg/day at day 28 end of the treatment, and at the end of recovery period day 56; decreased TP and ALB levels in females at 150 mg/kg/day on both day 28 and day 56, elevated TBIL levels in females at 150 mg/kg/day (although not statistically significant), increased ALP levels in females at 150 mg/kg/day on day 28, increased BUN levels in females at 150 mg/kg/day on both day 28 and day 56, increased CRE levels in females at 150 mg/kg/day on day 56, and increased TCH levels in females at 150 mg/kg/day on day 56. These changes suggest potential liver toxicity at the highest tested dose (150 mg/kg), particularly in female rats, with some effects persisting even after the recovery period. At the 37.5 mg/kg and 70 mg/kg dosing groups both male and females showed well reversibility. Male rats showed fewer significant changes in liver function parameters compared to females. This observation confirmed the body weight lose phenomenon that the female rats appeared more sensitive to SPRC than that of male rats.

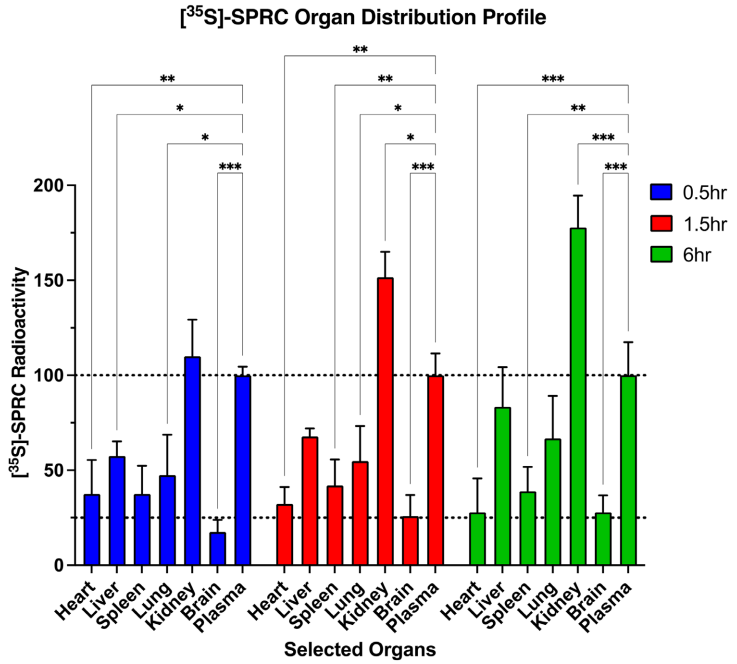


Figure 4. The distribution of [35S]-SPRC-derived radioactivity in the main organs of rats after oral administration of SPRC (25 μCi/mg, 75 mg/kg). The blue bars represent the 0.5-hour time point, the red bars represent the 1.5-hour time point, and the green bars represent the 6-hour time point. The radioisotope 35S-SPRC organ absolute counts were normalized by the corresponding time point of plasma concentration, with the plasma value set as 100. Statistical analysis was performed using two-way ANOVA with multiple comparisons. Data are presented as mean ± SD (n = 5). Statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), and * ($p < 0.001$).**

Table 2. The effects of intragastrically (ig) administered SPRC on blood biochemistry.

Item (unit)	Time (Day)	Control		37.5 mg/kg/d		70 mg/kg/d		150 mg/kg/d	
		Male	Female	Male	Female	Male	Female	Male	Female
ALT (nM/s/L)	d28	731 ± 66	510 ± 63	736 ± 121	622 ± 93	799 ± 194	1252 ± 599	1174 ± 377	1504 ± 653
	d56	909 ± 74	723 ± 166	948 ± 94	733 ± 127	939 ± 124	789 ± 255	1253 ± 235**	1480 ± 741

Continued

AST (nM/s/L)	d28	3053 ± 182	2796 ± 437	3118 ± 229	2681 ± 323	3251 ± 890	3369 ± 1120	2995 ± 830	3424 ± 738
	d56	2210 ± 459	2101 ± 378	2422 ± 856	2054 ± 496	2581 ± 676	2791 ± 1071	2710 ± 791	2996 ± 738
TP (g/L)	d28	47.4 ± 2.3	53.1 ± 1.9	47.9 ± 2.3	53.1 ± 1.5	50.6 ± 1.5	51.2 ± 1.9	46.3 ± 4.1	42.6 ± 4.7**
	d56	53.6 ± 3.0	58.6 ± 5.2	52.7 ± 2.0	62.2 ± 3.8	52.0 ± 2.7	59.2 ± 3.3	51.9 ± 1.6	49.3 ± 5.6**
ALB (g/L)	d28	32.4 ± 1.6	36.0 ± 1.0	32.8 ± 0.9	35.7 ± 0.7	34.0 ± 0.9	34.8 ± 0.7	31.8 ± 2.8	28.9 ± 3.1
	d56	32.8 ± 1.9	36.6 ± 3.7	32.3 ± 1.1	37.4 ± 2.4	31.7 ± 1.8	35.9 ± 2.1	32.0 ± 0.4	29.5 ± 3.4**
TBIL (μM/L)	d28	0.74 ± 0.47	1.28 ± 0.55	0.83 ± 0.58	0.97 ± 0.36	1.29 ± 0.36	2.00 ± 1.23	2.39 ± 2.29	10.24 ± 8.11
	d56	2.41 ± 0.60	2.46 ± 0.43	1.80 ± 0.52	2.92 ± 1.50	1.89 ± 0.55	2.65 ± 1.43	1.94 ± 0.88	4.92 ± 4.10
ALP (μM/s/L)	d28	2.37 ± 0.59	1.10 ± 0.18	2.08 ± 0.35	1.06 ± 0.20	3.05 ± 0.70	1.24 ± 0.45	2.43 ± 0.47	1.89 ± 0.52**
	d56	2.15 ± 0.77	1.19 ± 0.60	2.35 ± 0.36	1.16 ± 0.28	2.31 ± 0.61	1.07 ± 0.24	3.10 ± 0.67	3.16 ± 2.46
BUN (mM/L)	d28	6.61 ± 0.99	7.33 ± 1.72	6.14 ± 0.42	6.79 ± 1.65	6.18 ± 0.37	10.03 ± 6.05	6.93 ± 0.52	13.27 ± 4.11**
	d56	6.34 ± 0.68	6.20 ± 0.98	6.10 ± 1.02	6.74 ± 0.64	6.60 ± 0.66	6.75 ± 0.87	6.28 ± 0.74	9.72 ± 0.89**
CRE (μM/L)	d28	32.0 ± 6.1	35.7 ± 5.2	30.1 ± 3.7	30.9 ± 5.8	26.0 ± 3.2	31.5 ± 4.7	26.2 ± 2.0	26.8 ± 4.1
	d56	32.8 ± 2.8	33.7 ± 4.9	32.5 ± 5.1	35.5 ± 2.3	34.6 ± 5.5	39.7 ± 4.7	31.8 ± 4.9	46.2 ± 6.6**
TCH (mM/L)	d28	1.32 ± 0.13	1.78 ± 0.27	1.63 ± 0.67	1.78 ± 0.26	1.34 ± 0.29	1.95 ± 0.59	2.17 ± 0.87	2.17 ± 0.47
	d56	1.64 ± 0.35	1.74 ± 0.60	1.30 ± 0.22	1.99 ± 0.31	1.36 ± 0.10	1.87 ± 0.54	1.79 ± 0.72	2.83 ± 0.69**
TG (mM/L)	d28	0.35 ± 0.03	0.26 ± 0.03	0.37 ± 0.09	0.27 ± 0.03	0.40 ± 0.05	0.41 ± 0.20	0.89 ± 0.54	0.71 ± 0.35
	d56	0.38 ± 0.03	0.31 ± 0.08	0.35 ± 0.05	0.30 ± 0.05	0.33 ± 0.04	0.30 ± 0.06	0.60 ± 0.33	0.89 ± 0.44
CK (μM/s/L)	d28	9.31 ± 1.33	8.82 ± 0.97	10.20 ± 0.93	8.48 ± 1.73	10.72 ± 3.15	7.55 ± 2.94	8.63 ± 2.59	5.88 ± 2.27
	d56	8.04 ± 2.73	7.82 ± 1.72	9.73 ± 6.40	7.18 ± 1.99	10.31 ± 3.59	8.89 ± 2.08	10.32 ± 3.34	7.07 ± 1.06
LDH (μM/s/L)	d28	38.49 ± 3.33	36.07 ± 6.38	39.92 ± 2.52	34.17 ± 6.49	40.48 ± 9.49	32.13 ± 9.07	36.20 ± 10.83	28.16 ± 6.92
	d56	29.90 ± 11.43	29.77 ± 6.17	31.69 ± 13.68	26.25 ± 6.47	34.63 ± 11.27	32.48 ± 6.84	32.46 ± 11.00	32.88 ± 5.35

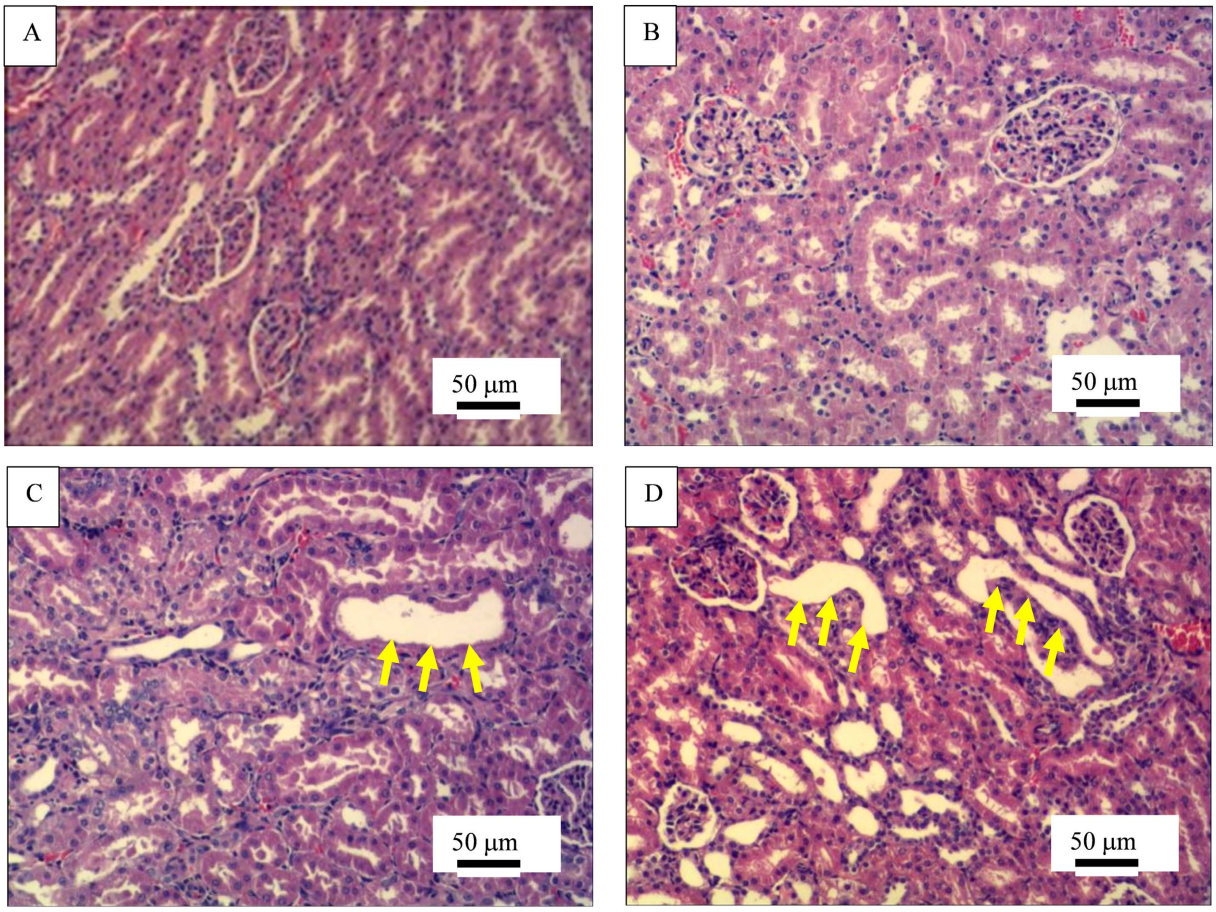
Parameters in male rats during the toxicity study. The data are expressed as mean ± standard deviation (SD), with a sample size of 5 animals per group. The blood biochemistry parameters assessed include: Alanine Aminotransferase (ALT); Total Bilirubin (TBIL); Albumin (ALB); Alkaline Phosphatase (ALP); Aspartate Aminotransferase (AST) 6. Blood Urea Nitrogen (BUN); Creatinine (CRE); Total Cholesterol (TCH); Triglycerides (TG); Creatine Kinase (CK); Lactate Dehydrogenase (LDH). Statistical significance is denoted by ** ($P < 0.01$), indicating a significant difference between the SPRC-treated groups and the control group for the corresponding blood biochemistry parameter. These blood biochemistry parameters help assess the potential effects of SPRC on various organ systems: Liver function: ALT, TBIL, ALB, ALP, and AST; Kidney function: BUN and CRE; Lipid metabolism: TCH and TG; Muscle and tissue damage: CK and LDH. Monitoring these parameters provides valuable information about the safety and potential toxicity of SPRC during the administration in male and female rats.

Histopathological examination revealed tubular dilation in the kidneys (**Figure 5**) and inflammatory cell infiltration surrounding central hepatic venules (**Figure 6**) in the mid- and high-dose groups after 28 days of treatment. These changes exhibited dose-dependent severity and frequency and were absent in low-dose and control animals. Partial recovery was evident as tubular and hepatic lesions persisted after the off-dose period in the high-dose group but not at lower doses. Thus, SPRC-mediated nephrotoxicity and hepatotoxicity were reversible, though higher exposure levels caused sustained tissue changes. Overall, histological findings indicated dose-responsive target organ toxicity that could regress upon cessation of dosing at

lower doses.

In addition, safety pharmacology assessments with utilizing mice and beagle dogs for evaluating central neuron, cardiovascular and respiratory system in safety, respectively. For evaluating central neuron system, single-dose intragastric SPRC up to 200 mg/kg (100 mg/kg in rat equivalent) in mice revealed no compound-related adverse effects on the nervous system by observing for spontaneous activity test and pole climbing ability test (reference the Data in Brief article). Similarly, for evaluating cardiovascular and respiratory system in safety, a single dose of i.g. SPRC 4, 8, and 16 mg/kg in beagle dogs (14, 27, 54 mg/kg in rat equivalent, respectively) had no noteworthy effects on systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, heart rhythm, ECG P wave voltage, T wave voltage, QRS time, PR interval, and QT interval in Beagle dogs. There were no significant effects on phase ST segment, respiratory frequency, rhythm and amplitude (reference the Data in Brief article).

Based on the genotoxicity battery results: 1) Salmonella typhimurium reverse mutation assay (Ames test): The number of reverted colonies was less than 2 times the number in the blank control group. No dose-response relationship was observed; 2) Mouse bone marrow micronucleus test: No inhibitory effect of propargylcysteine on mouse bone marrow at any tested dose. Positive control group showed statistically significant increase in micronucleus induction rates compared to negative control ($P < 0.05$). Test substance at doses of 429.25, 858.5, and 1717.0 mg/kg showed no statistically significant differences in micronucleus induction rates compared to negative control ($P > 0.05$); 3) Chromosome aberration test in CHO cells: Positive control induced a significant increase in chromosome aberration rate compared to solvent control ($P < 0.05$). SPRC at 750, 1500, and 3000 μg/ml showed chromosome aberration rates < 5% under various conditions (4h, +S9; 4h, −S9; 24h, −S9), no statistical significance compared to solvent control group ($P > 0.05$) (reference the Data in Brief article).



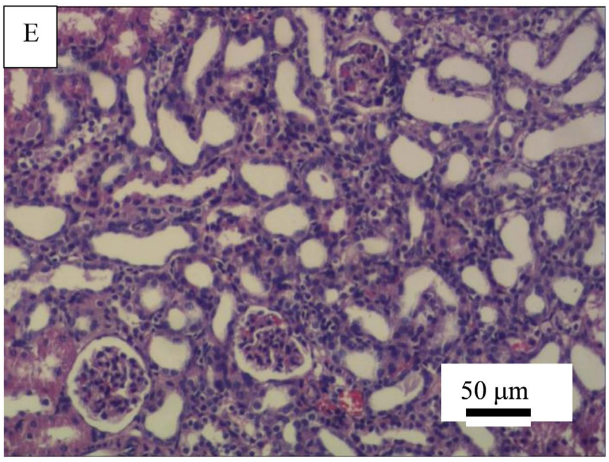
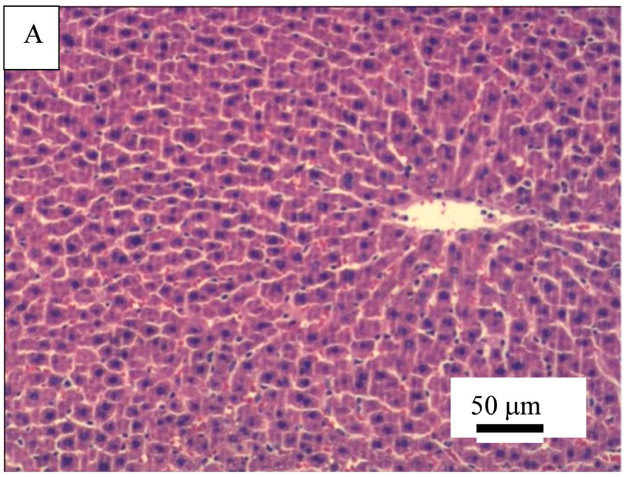
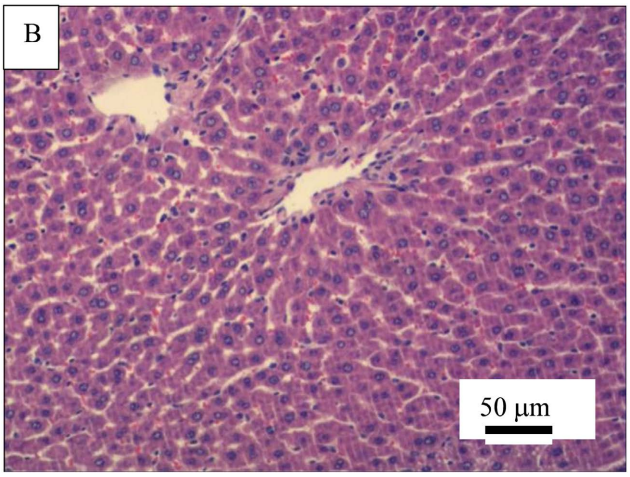


Figure 5. The histopathology of the kidney cortex in female rats following a 28-day oral administration of S-propargyl-cysteine (SPRC) at various doses. A) Vehicle control group (H&E, 100× magnification): The image shows the normal renal architecture, consisting of glomeruli, tubules, and interstitium. B) 37.5 mg/kg/day SPRC group (H&E, 100× magnification): No pathological changes were observed in the kidney cortex compared to the control group. C) 75 mg/kg/day SPRC group (H&E, 100× magnification): Tubular epithelial dilation is indicated by arrows, representing a pathological change in the kidney cortex at this dose. D) 150 mg/kg/day SPRC group (H&E, 100× magnification): Severe tubular dilation is denoted by arrows, indicating a more pronounced pathological change in the kidney cortex at the highest dose tested. E) 150 mg/kg/day SPRC group at day 56, following the recovery period (H&E, 100× magnification): This image shows the kidney histology of a female rat from the 150 mg/kg/day SPRC group after a 28-day recovery period. The marked tubular dilation (indicated by arrows) was partially resolved compared to the end of the treatment period. However, some degree of tubular dilation persisted, suggesting that additional recovery time may be necessary for complete restoration of normal tubular structure. The histopathological findings in the kidney cortex demonstrate a dose-dependent effect of SPRC on renal tubular structure, with higher doses (75 and 150 mg/kg/day) causing tubular dilation. The partial recovery observed in the 150 mg/kg/day group after a 28-day recovery period indicates the potential for reversibility of these changes, although complete recovery may require more time.





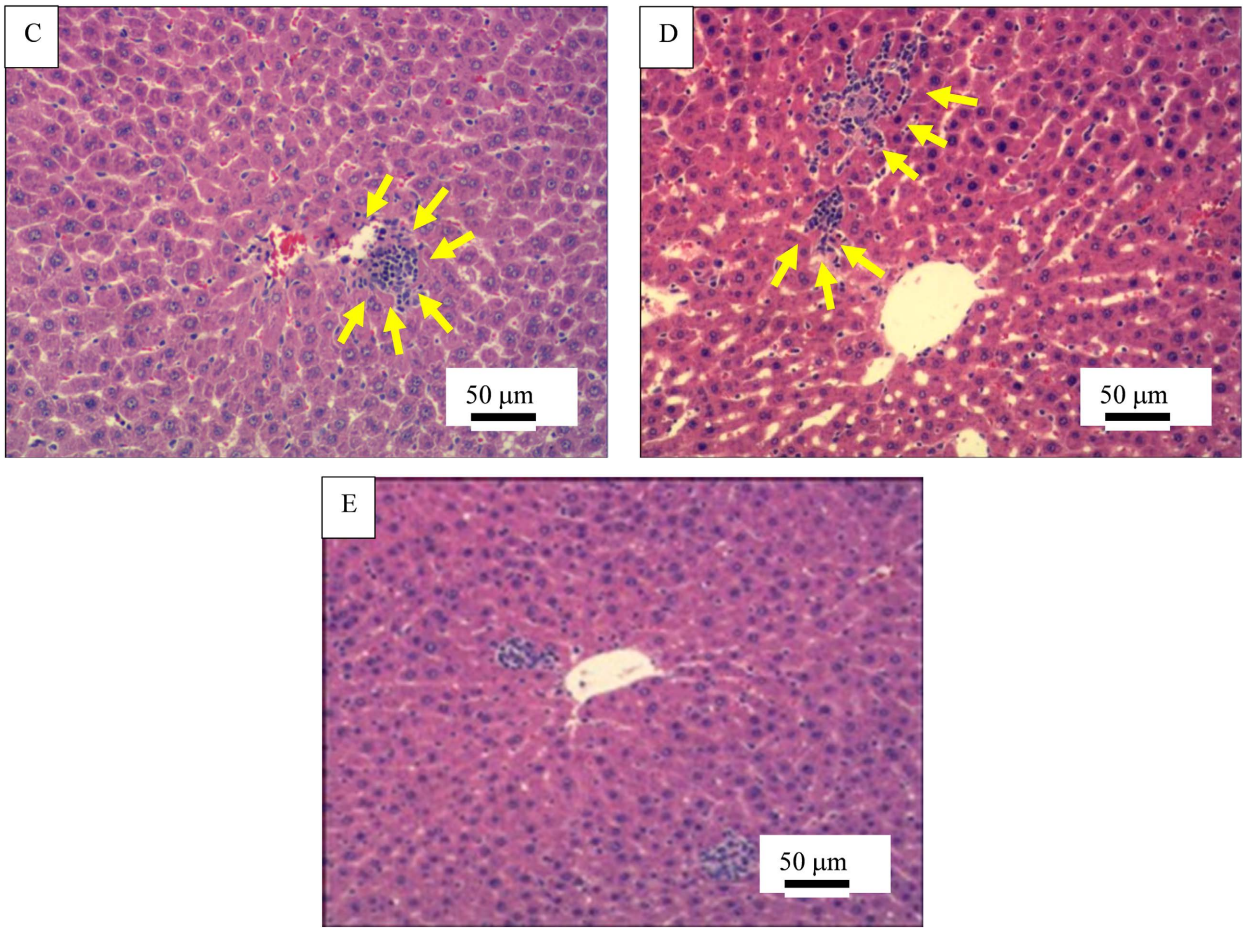


Figure 6. The histopathology of the liver from rats following a 28-day oral administration of Spropargyl-cysteine (SPRC) at various doses. Vehicle control female (H&E, 100× magnification): The image shows normal hepatic lobule architecture, serving as a reference for comparison with the treated groups. B) 37.5 mg/kg/day SPRC female (H&E, 100× magnification): No treatment-related changes were observed in the liver at this dose, indicating that SPRC did not induce any notable histopathological alterations. C) 75 mg/kg/day SPRC male (H&E, 100× magnification): Pericentral inflammatory cell infiltration is indicated by arrows, representing a pathological change in the liver at this dose. D) 150 mg/kg/day SPRC male (H&E, 100× magnification): Marked inflammatory cell infiltration around central veins is denoted by arrows, indicating a more severe pathological change in the liver at the highest dose tested. E) 150 mg/kg/day SPRC male at day 56, following the recovery period (H&E, 100× magnification): This image shows the liver histology of a male rat from the 150 mg/kg/day SPRC group after a 28-day recovery period. The marked inflammatory cell infiltration around the central veins (indicated by arrows) was significantly reduced compared to the end of the treatment period. However, some degree of infiltration persisted, suggesting that additional recovery time may be necessary for complete resolution of the inflammatory response. The histopathological findings in the liver demonstrate a dose-dependent effect of SPRC on hepatic inflammation, with higher doses (75 and 150 mg/kg/day) causing pericentral inflammatory cell infiltration. The significant reduction in inflammation observed in the 150 mg/kg/day group after a 28-day recovery period indicates the potential for reversibility of these changes. However, the persistence of some infiltration suggests that complete recovery may require more time.

6. CONCLUSION

Based on body weight changes, clinical chemistry, and histopathological findings, SPRC was administered via oral gavage at doses of 0, 37.5, 75, and 150 mg/kg/day to rats continuously for 28 days (n=10 per group) followed by a 28-day recovery period (n=5 per group). The no observed-adverse-effect level (NOAEL) was 37.5 mg/kg/day. At 75 mg/kg/day, treatment-related adverse effects were observed establishing this as the minimum toxic dose. Severe toxicity and lethality occurred at the high dose of 150 mg/kg/day. All drug-induced findings showed greater severity in female rats compared to males at the same doses. The primary target organs of toxicity were the digestive system (gastrointestinal tract and liver), hematopoietic system (peripheral blood and bone marrow), and kidneys. The high-dose mediated body weight gain losses and organ toxicity were partially reversible during the recovery phase. Repeat-dose preclinical studies at the NOAEL revealed no test article-related adverse signals. Thus, there are no significant safety concerns for the intended therapeutic use at the established NOAEL based on this preclinical toxicology assessment.

Due to single doses safety pharmacology studies of SPRC 50, 100, and 200 mg/kg had no significant effect on the spontaneous activity test and pole climbing ability test of mice had no obvious effect on the nervous system of mice; and corresponding single i.g. administration of SPRC 4, 8, and 16 mg/kg had no noteworthy effect on the cardiovascular system and respiratory system of Beagle dogs. The studies demonstrated that SPRC has sufficient safety pharmacology property.

The standardized genetic toxicology study battery, conducted in compliance with GLP, yielded consistently negative results for endpoints such as bacterial mutagenicity and chromosomal damage. 1. Ames test (bacterial mutagenicity): Propargyl cysteine was tested at doses of 5000, 3000, 300, 30, 3, and 0.3 g/plate with and without metabolic activation (S9). No mutagenicity observed in S. typhimurium strains TA97, TA98, TA100, TA102, and TA1535; 2. Mouse bone marrow micronucleus test (chromosomal damage): SPRC tested at doses of 429.25, 858.5, and 1717.0 mg/kg. No micronucleus-inducing effect on bone marrow polychromatic erythrocytes of ICR mice; 3. Chromosome aberration test in CHO cells (chromosomal damage): SPRC tested at doses of 750, 1500, and 3000 μg/ml. No teratogenic effect on CHO cell chromosomes under non-metabolic activation and metabolic activation conditions. Thus, SPRC did not exhibit mutagenic, micronucleus-inducing, or teratogenic effects in the standardized genetic toxicology study battery.

The key pharmacokinetic parameters of SPRC in rats following a single oral administration at 75 mg/kg are as follows: C_{max} (59.0 ± 9.9 μg/mL), representing the peak plasma concentration; T_{max} (1.3 ± 0.5 hours), indicating the time to reach peak plasma concentration; $T_{1/2}$ (3.0 ± 0.9 hours), the time for plasma concentration to decrease by half during elimination; CL (2.8 ± 0.8 mL/min/kg), the volume of plasma cleared of SPRC per unit time; Vd, area (0.73 ± 0.27 L/kg), the apparent volume of distribution based on the area under the curve; AUC_{0-t} (457.1 ± 137.2 mg/l·h), the area under the plasma concentration-time curve from time 0 to the last measurable concentration; $AUC_{0-\infty}$ (468.4 ± 136.8 mg/l·h), the area under the plasma concentration-time curve from time 0 to infinity; and F (97.0%), the fraction of the administered dose that reached the systemic circulation. These parameters provide insights into SPRC's absorption, distribution, metabolism, and elimination in rats after oral administration, with the high bioavailability (97.0%) suggesting good absorption from the gastrointestinal tract and the short elimination half-life (3.0 ± 0.9 hours) indicating rapid clearance from the body.

7. DISCUSSION

As research on SPRC continues to unfold, it may soon carve a niche for itself in the ever-evolving drug discovery landscape. SPRC emerged as the lead candidate that balanced potency, selectivity, and drug-like properties through the preclinical stage. We aim to gather robust data that support its clinical potential. SPRC's mechanism of action is intricately linked to its ability to modulate H_2S levels, thereby influencing pathways related to oxidative stress, inflammation, and cellular apoptosis [3, 6, 9, 12, 23, 24]. The goal is to develop safe and effective therapies that harness SPRC's unique mechanisms for the treatment of various diseases.

While the preclinical data set presented here may not be all-encompassing, it encompasses a significant portion of the information needed to illustrate our comprehensive strategy for advancing a drug candidate from discovery to clinical development. This approach offers a detailed roadmap, outlining the essential steps and assessments necessary to guide a promising compound through the preclinical phase, ultimately preparing it for evaluation in human clinical trials. By providing this informative framework, we aim to enhance understanding of the complex process involved in drug development and support the successful progression of novel therapeutic agents.

Our focus is on the principal of toxicity in the organs, which is crucial for safety evaluation and translational readiness for clinical research and development. Rather than discussing each parameter, such as liver functional chemistry data, in detail, we prioritize the overall assessment of organ toxicity to ensure the compound's safety profile is suitable for advancing to human trials.

SPRC is expected to have reasonable absorption from oral administration, with a 97.0% bioavailability across preclinical species [21, 22] providing insights into the feasibility of clinical oral dosing. SPRC's linear pharmacokinetic (PK) behavior indicates that its serum concentrationtime profiles are consistent with a one-compartment pharmacological model. The linear relationship between SPRC dose and observed exposure metrics (e.g., Cmax and AUC) over a therapeutic dose range implies that it follows dose-proportional pharmacokinetics without saturation of processes influencing absorption or clearance pathways (**Table 3**) [21]. The one compartment model further suggests that the distribution of SPRC into tissues achieves rapid equilibration relative to the overall elimination half-life. These properties are favorable indications of predictable pharmacokinetic behavior for SPRC as the dose level or frequency is adjusted within a therapeutic range. The consistently proportional relationship between dosing, systemic exposure, and assumed kinetics enables straightforward modeling of expected pharmacokinetic responses over a range of administration scenarios without complicated distribution or clearance nonlinearities.

The high oral bioavailability and linear pharmacokinetics of SPRC are advantageous for its clinical development. These characteristics suggest that SPRC's absorption and distribution are not significantly affected by dose-dependent factors, allowing for more predictable exposure and therapeutic effects. The one-compartment model simplifies the understanding of SPRC's pharmacokinetic behavior, as it assumes rapid and unique distribution features throughout the body. This simplification facilitates the prediction of SPRC's concentration-time profile and the adjustment of dosing regimens to maintain therapeutic levels. Moreover, the dose-proportional pharmacokinetics of SPRC indicate that its exposure metrics increase linearly with increasing dose, without saturation of absorption or clearance processes within the therapeutic range. This linearity is crucial for establishing a clear relationship between dose and response, enabling the optimization of dosing strategies for clinical trials. The absence of complex distribution or clearance nonlinearities further enhances the predictability of SPRC's pharmacokinetic behavior, reducing the risk of unexpected exposure-related issues during clinical development.

The volume of distribution (Vd) of SPRC is 0.73 L/kg, which is comparable to total body water (≈0.6 L/kg). This implies good permeability into extracellular fluid and extensive distribution into body fluids. SPRC's wide distribution in blood and tissues may account for its diverse biological activities and broader potential clinical indications. Interestingly, its kidney accumulation was nearly double plasma levels over the first six hours, suggesting the involvement of a special energy-spending transporter system associated with the higher concentration in the kidneys compared to that of the plasma. As SPRC and its metabolites were not detected in feces, the kidney likely plays a major role in reabsorption and elimination [19]. Time- and dose-dependent kidney accumulation of SPRC with proportional toxicity has been observed, comprising tubule dilation at mid and high doses, but no signals at low doses (**Figure 4**). The distribution-toxicity relationship warrants further monitoring of biomarkers like Kim-1, Clusterin, and Cystatin C, as the FDA recognizes their utility in tracking compound-induced kidney injury during clinical dose escalation and risk evaluation [25]. In contrast, the distribution in the heart and brain was barely a quarter of that in the kidneys over the first 6 hours, indicating fewer SPRC allocated in these two key organs. This finding was supported by safety pharmacology data, which showed no toxic signals in the heart and brain even at the mid-dose that

exhibited toxic signals in other organs with higher SPRC distribution, such as the kidney and liver.

Table 3. The pharmacokinetic parameters in rats (mean ± SD, n = 6) presented in Table 2.

Parameters	i.g.
Dose (mg/kg)	75
Cmax (μg/mL)	59.0 ± 9.9
Tmax (h)	1.3 ± 0.5
$T_{1/2}$ (h)	3.0 ± 0.9
CL (mL/min/kg)	2.8 ± 0.8
Vd, area (L/kg)	0.73±0.27
AUC0−t (mg/l h)	457.1 ± 137.2
AUC0-∞ (mg/l h)	468.4 ± 136.8
F (%)	97.0

Intragastric (i.g.) administration: The method of drug administration through the stomach. C_{max}: The maximum plasma concentration achieved after drug administration. T_{max}: The time at which the maximum plasma concentration (C_{max}) is observed. $T_{1/2}$: The elimination half-life, which represents the time required for the plasma concentration to decrease by half. CL: Clearance, a measure of the volume of plasma cleared of the drug per unit time. Vd: The volume of distribution, which represents the theoretical volume needed to contain the total amount of drug at the same concentration as in the plasma. AUC_{0-t}: The area under the plasma concentration-time curve from time zero to a specific time point (t), a measure of drug exposure over that time. $AUC_{0-\infty}$: The area under the plasma concentration-time curve from time zero to infinity, representing the total drug exposure. F: Bioavailability, the fraction of the administered dose that reaches the systemic circulation unchanged. These pharmacokinetic parameters provide essential information about the absorption, distribution, metabolism, and elimination of the drug in rats.

Interestingly, the observed toxicity did not always proportionally relate to the concentration of SPRC tissue distribution and the corresponding organ weight loss. Stronger inflammation signals were observed in the liver by pathological examination (**Figure 5**) and blood chemistry (**Table 2**), with relatively higher toxicity and a significant decrease in liver weight compared to the kidneys. The consistently lower concentration of SPRC in the brain over the first six hours reveals its difficulty in penetrating the blood-brain barrier, despite its low molecular mass. Given SPRC's broad range of biological effectiveness in preclinical settings, the PK profile, coupled with its unique properties, helps predict effective concentrations for pharmacological efficacy and safety.

Moreover, the IC50 for SPRC is highly dependent on the specific assay conditions, making it difficult to provide a general value. More experimental context would be needed to interpret any IC50 result. A previous study demonstrated that 1 mM SPRC produced 18% inhibition of viability, suppressed colony forming and migration ability, prevented nuclear factor-kB (NF-kB) activation assessed by NF-kB p65 phosphorylation and IkBa degradation, suppressed LPS induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and reduced intracellular reactive oxygen species (ROS) production in vitro [23, 26]. Given

the assumption of oral bioavailability as low as 50% in humans, due to the absence of ongoing clinical trials, the anticipated human dose (AHD) was conservatively estimated to be 0.7 mg/kg [27]. This level of potency was maintained across cell lines from multiple species and tissue origins, including disease-relevant primary human cells.

To fully characterize its safety profile, SPRC underwent a comprehensive battery of good laboratory practice (GLP) toxicology studies in rats and dogs. These studies demonstrated a favorable safety pharmacology profile that supports further development of SPRC as a promising drug candidate.

The in vitro studies provide valuable insights into the potential therapeutic effects of SPRC, demonstrating its ability to inhibit cell viability, suppress colony formation and migration, and modulate key signaling pathways involved in inflammation and oxidative stress. However, it is important to note that the IC50 values obtained from these studies are highly dependent on the specific experimental conditions and may not be directly translatable to *in vivo* settings.

The assumption of 50% oral bioavailability in humans, in the absence of clinical trial data, allows for a conservative estimation of the anticipated human dose. This approach helps to minimize the risk of overestimating the potential therapeutic effects and ensures a cautious interpretation of the preclinical data.

The consistent potency of SPRC across various cell lines, including those derived from disease relevant primary human tissues, strengthens its potential as a therapeutic agent. This broad spectrum activity suggests that SPRC may have applications in multiple disease areas, increasing its versatility as a drug candidate.

The comprehensive GLP toxicology studies conducted in rats and dogs provide a robust assessment of SPRC's safety profile. The favorable safety pharmacology results obtained from these studies are crucial for supporting the further development of SPRC and its progression to clinical trials.

In a repeat-dose toxicity study complying with GLP for a 28-day treatment period followed by a 28-day recovery period, the no-observed-adverse-effect level (NOAEL) for SPRC was determined to be 37.5 mg/kg. Based on the NOAEL in the rat 28-day toxicity study, the human equivalent dose (HED) is estimated at approximately 6.0 mg/kg or 360 mg for a 60 kg adult [22]. To determine the maximum recommended starting dose (MRSD) for sensitive populations, a safety factor of 1/10 is applied, resulting in a minimal anticipated biological effect level (MABEL) of 36 mg for a 60 kg adult for Single Ascending Dose (SAD) and Repeat Ascending Dose (RAD) approaches.

The therapeutic window is calculated by dividing the HED (6.0 mg/kg) by the anticipated human dose (AHD) (0.7 mg/kg), yielding an approximate 8.5-fold therapeutic window [27]. This estimate suggests that SPRC has a favorable safety profile with a therapeutic window of up to 8.5-fold in humans over the HED of 36 mg/day for an adult.

Key safety studies, safety pharmacology, revealed no adverse signals at the NOAEL. Assessing reversibility after the end of dosing is also crucial for understanding chronic versus acute liability concerns, which will be important for follow-up regulatory discussions. Genetic toxicology studies were uniformly negative and complied with GLP, providing satisfactory results. SPRC is considered a compound with promising druggability potential in the preclinical stage of drug development. Its multifaceted mechanism of action, coupled with preclinical research findings, suggests that it holds significant promise as a therapeutic agent for a range of diseases. However, further research and development efforts are needed to fully realize its clinical potential and identify SPRC-specific indications.

The GLP-compliant repeat-dose toxicity study, which included a recovery period, provides valuable information about the safety profile of SPRC. The NOAEL of 37.5 mg/kg in rats and the estimated HED of 6.0 mg/kg (360 mg for a 60 kg adult) demonstrate a favorable safety margin. The application of a safety factor to determine the MRSD for sensitive populations further enhances the confidence in SPRC's safety profile.

The calculation of the therapeutic window, which is estimated to be approximately 8.5-fold in humans, suggests that SPRC has a range between the effective dose and the dose that may cause adverse effects. This therapeutic window is a desirable characteristic for a drug candidate, as it allows for flexibility in dosing and reduces the risk of adverse events.

The absence of adverse signals in safety pharmacology, at the NOAEL further supports the favorable safety profile of SPRC. Assessing reversibility after the end of dosing is essential for understanding the potential for chronic or acute liabilities, which will be important for discussions with regulatory authorities.

The negative results in genetic toxicology studies, which were conducted in compliance with GLP, provide additional assurance regarding the safety of SPRC. These studies help to rule out potential genotoxic effects that could raise concerns about long-term safety.

Overall, the preclinical data suggest that SPRC is a promising drug candidate with a favorable safety profile and a wide therapeutic window. Its multifaceted mechanism of action and the positive preclinical research findings indicate its potential as a therapeutic agent for various diseases. However, further research and development efforts are necessary to fully understand its clinical potential and identify specific indications for which SPRC may be most effective. As SPRC progresses through the drug development pipeline, additional studies will be required to further characterize its safety, efficacy, and optimal dosing regimens in humans. These studies may include clinical trials in healthy volunteers and patient populations, as well as long-term safety and efficacy studies. The data generated from these studies will be critical for making informed decisions about the future development and potential commercialization of SPRC as a novel therapeutic agent.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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