

**A Phase II clinical trial evaluating the safety and efficacy of AS1501 for injection in the
treatment of patients with acute-on-chronic liver failure (ACLF)
Clinical Trial Protocol**

Plan Number: AS1501-CTP-Ⅱ-01

version number : V3.1

Version Date: 2025/12/23

Principal Investigator: Professor Chen Jun

Group Leading Unit: Shenzhen Third People's Hospital

Applicant: Shenzhen Zhongke Aishen Pharmaceutical Co.,
Ltd.

contract research organization : Kunling Enterprise Management Shanghai Co.,
Ltd.

declaration of secrecy

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Page with the signatures of the principal investigators

We have reviewed and confirmed this protocol (Protocol Number: AS1501-CTP- II -01, Version Number: v3.1, Version Date: December 23,2025). We agree to fulfill our respective obligations in accordance with the Helsinki Declaration, Good Clinical Practice (GCP), relevant laws and regulations, and this study protocol, and are responsible for conducting the clinical trial.

clinical research unit :

Sign of the principal investigator:

sign one's name

date

Applicant Signature Page

We have reviewed and confirmed this protocol (Protocol Number: AS1501-CTP-II-01, Version Number: v3.1, Version Date: 2025/12/23). We agree to fulfill our respective obligations in accordance with the Helsinki Declaration, Good Clinical Practice (GCP), relevant laws and regulations, and this study protocol, including initiating, applying for, organizing, funding this clinical trial, and conducting audit activities related to its implementation.

Sponsor: Shenzhen Zhongke Aishen Pharmaceutical Co., Ltd.

Sponsor Representative Signature:

sign one's name

date

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Abbreviation List

Abbreviations and Terms	Full English Name	Chinese Explanation
AARC	Asian Acute-on-Chronic Liver Failure Research Working Group	ACLF Research Alliance of the Asia-Pacific Liver Disease Research Society
ACLF	Acute-on-chronic liver failure	Slow-onset acute liver failure
ADA	Anti drug antibody	Drug-resistant antibodies
AE	Adverse event	adverse event
AESI	Adverse Event of Special Interest	Adverse events of particular concern
AIH	Autoimmune hepatitis	autoimmune hepatitis
ALB	Albumin	albumin
ALF	Acute liver failure	acute hepatic failure
ALT	alanine aminotransferase	Alanine aminotransferase
ALP	alkaline phosphatase	alkaline phosphatase
ANC	absolute neutrophil count	Neutrophil count
APTT	activate partial thromboplastin time	Activated partial thromboplastin time
AST	aspartate aminotransferase	Aspartate aminotransferase
AUC	Area under curve	Area under the drug-time curve
AUC _{last}	Area Under the Curve from time zero to the last measurable concentration	The area under the curve from the start of administration to the last measurable concentration point
AUC _{ss}	Area Under the Plasma Concentration-Time Curve at Steady State	Area under the steady-state plasma concentration-time curve
BMI	Body Mass Index	Body mass index
BUN	blood urea nitrogen	Urea nitrogen
Ca	Calcium	calcium
C _{max}	Maximum concentration	Maximum plasma drug concentration

Css-max	Maximum concentration at Steady State	Steady-state plasma peak concentration
Css-min	Minimum concentration at Steady State	Steady-state plasma valley concentration
Cl	Chlorine	chlorine
CL	Plasma clearance	plasma clearance
Cr	Creatinine	creatinine
CRF	case report form	Case Report Form
CRP	C-reactive protein	C reactive protein
CTCAE	Common Terminology Criteria for Adverse Events	General Terminology Standards for Adverse Events
DAMP	Damage - associated molecular patterns	injury-related pattern molecules
DBil	Direct bilirubin	bilirubin direct
DISC	Death inducing signaling complex	Death-inducing signaling complex
SMC	Safety Monitoring Committee	Safety Inspection Committee
DNA	Deoxyribonucleic acid	deoxyribonucleic acid
EDC	Electronic Data Capture System	Electronic Data Acquisition System
ELISA	Enzyme-linked immunosorbent assay	Enzyme-linked immunosorbent assay (ELISA)
FAS	full analyse set	Full Analysis Set
Fas L	Fas ligand	Fas part
GCP	Good Clinical Practice	Clinical Trial Management Standards
GGT	Glutamyltransferase	γ- glutamyltranspetidase
h	hour	hour
Hb	Hemoglobin	hemoglobin
HBcAb	Hepatitis B central antibody	Hepatitis B core antibody
HBsAb	Hepatitis B surface antibody	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen	HBsAg
HBV	Hepatitis B virus	hepatitis B virus

HCV	Hepatitis C virus	HCV
HDS	Herbal and dietary supplement	Herbal Medicines and Dietary Supplements
HIV	human immunodeficiency virus	human immunodeficiency virus
IAIHG	International autoimmune hepatitis group	International Autoimmune Hepatitis Group
INR	International Normalized Ratio	international normalized ratio
ITT	intend to treat	Intentional Therapy
K	potassium	potassium
LVEF	Left Ventricular Ejection Fractions	left ventricular ejection fraction
MedDRA	Medical Dictionary for Regulatory Activities	International Dictionary of Medical Terminology
MELD	Model For End Stage Liver Disease	End-stage liver disease model
Mg	Magnesium	magnesium
Min	minute	minute
MRI	Magnetic resonance imaging	magnetic resonance
MSD	Supersensitive Electrochemiluminescence	Electrochemiluminescence method
Na	Sodium	sodium
NAFLD	Nonalcoholic fatty liver disease	Non-alcoholic fatty liver disease
NOAEL	no observable adverse effect level	Dose without observed toxic effects
P	Phosphorus	phosphorus
PAMP	Pathogen - associated molecular patterns	Pathogen-related pattern molecules
PD	Pharmacodynamics	pharmacodynamics
PLT	Platelet	blood cells
PK	pharmacokinetics	pharmacokinetics
PT	prothrombin time	prothrombin time
PTA	Prothrombin activity	Prothrombin activity
RBC	red blood cell	RBC
RNA	Ribonucleic acid	ribonucleic acid

RP2D	recommended phase 2 dose	Recommended dosage for Phase 2 clinical trial
RUCAM	the Roussel Uclaf Causality Assessment Method	RUCAM Method for Evaluating Causal Relationships
SAE	serious adverse event	Serious adverse events
SOP	Standard Operation Procedure	standard practice
SS	Safety Analysis set	Safety Analysis Set
TBil	Total bilirubin	total bilirubin
TCM	Traditional Chinese Medicine	Traditional Chinese Medicine
TEAE	Treatment-Emergent Adverse Events	Adverse events occurring after medication administration
Tmax	Time to Maximum Concentration	Peak Reach Time
TNF-α	Tumor Necrosis Factor-α	TNF -α
TRAIL	TNF-related apoptosis inducing ligand	TNF-related apoptosis-inducing ligand
TRAIL-DR5	TNF-related apoptosis inducing ligand -death receptor 5	TNF-related apoptosis-inducing ligand-death receptor 5
ULN	upper limit of normal	Upper limit of normal value
UREA	——	urea
WBC	white blood cell	leucocyte count

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1. scenario summary

1.1. Solution Overview

Plan Number	AS1501-CTP- II-01
Research Title	A Phase II clinical trial evaluating the safety and efficacy of AS1501 for injection in the treatment of patients with acute-on-chronic liver failure (ACLF)
Version Number/Date	V3.1/2025-12-23
Registration Category	Category 1 therapeutic biological products
Stage of Research	II designated time
Applicant	Shenzhen Zhongke Aishen Pharmaceutical Co., Ltd.
Principal Investigator	Professor Chen Jun
Clinical Trial Institution	Shenzhen Third People's Hospital
purpose of research	IIa designated time fundamental purpose : • To evaluate the safety and tolerability of AS1501 for injection in patients with early-stage acute-on-slow-onset liver failure (ACLF) and to explore an optimal dosing regimen. • Determine the recommended dosage and administration frequency for Phase IIb studies. Secondary objectives: • Preliminary evaluation of the efficacy, immunogenicity, and pharmacokinetic (PK) characteristics of AS1501 for injection in patients with early acute lymphoblastic leukemia (ACLF). • This preliminary study investigated changes in serum TRAIL (TNF-related apoptosis-inducing ligand) and NK cell membrane-type TRAIL expression levels before and after administration of AS1501 for injection in early-stage ACLF patients. Exploratory Purpose: • Preliminary investigation into the cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo. IIb designated time fundamental purpose : • Assess the efficacy of AS1501 for injection in treating subjects with ACLF. Secondary objectives:

	- To evaluate the safety, pharmacokinetic (PK) characteristics, and immunogenicity of AS1501 for injection in patients with acute myeloid leukemia (AML). - To investigate the changes in serum TRAIL levels and NK cell membrane-type TRAIL expression before and after administration of AS1501 for injection in ACLF patients. Exploratory Purpose: - Preliminary investigation into the cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.
Study endpoint	IIa designated time Primary endpoint: - The incidence rates of various adverse events (AEs) and serious adverse events (SAEs), as well as the incidence rates of AEs and SAEs associated with the study product. Secondary endpoint: - Changes in the End-Stage Liver Disease Model (MELD) score and the Asian Association for Research on Liver Diseases (AARC)-CLF study alliance scores from baseline at the end of AS1501 treatment. - Survival rate of ACLF patients at the end of AS1501 treatment. - Improvement rate of ACLF patients at the end of AS1501 treatment; Criteria for improvement: ① Significant improvement in clinical symptoms such as fatigue, poor appetite, abdominal distension, and bleeding, with resolution of hepatic encephalopathy; ② Marked improvement in signs such as jaundice and ascites; ③ Significant improvement in liver function parameters (TBIL <5 ULN, PTA > 40%, or INR <1.5). - The proportion of ACLF patients with normal serum biochemical parameters (ALT, AST, Tbil, and PTA) at the end of AS1501 treatment. - The incidence of switching to other treatment modalities (artificial liver, liver transplantation, cell therapy, etc.). - Pharmacokinetic parameters of AS1501 after single and multiple administrations, including: apparent volume of distribution (Vd/f), plasma clearance (CL/F), plasma elimination half-life (t1/2), plasma peak concentration (Cmax), time to peak (Tmax), area under the plasma concentration-time curve (AUC), steady-state peak plasma concentration (Css-max), steady-state time to peak (Tss-max), steady-state trough plasma concentration (Css-min), area under the steady-state plasma concentration-time curve (AUCss), and accumulation ratio

	(Rac). 7) Immunogenicity-related indicators, such as ADA antibodies. 8) Preliminary investigation into the changes in serum TRAIL and NK cell membrane-type TRAIL expression levels before and after administration of AS1501 for injection in ACLF patients. Exploratory endpoints: 1) Preliminary investigation into cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo. IIb designated time Primary endpoint: 1) Survival rate of ACLF patients on day 28 after the first dose administration. Secondary endpoint: 1) Survival rates of ACLF patients at weeks 8 and 12 after the first dose administration. 2) Improvement rates in ACLF patients at weeks 4, 8, and 12 after the first administration. Criteria for improvement: ① Significant alleviation of clinical symptoms such as fatigue, poor appetite, abdominal distension, and bleeding, with resolution of hepatic encephalopathy; ② Marked improvement in signs such as jaundice and ascites; ③ Significant improvement in liver function parameters (TBIL <5 ULN, PTA> 40%, or INR <1.5). 3) The proportion of ACLF patients with normal serum biochemical response rates (ALT, AST, Tbil, PTA all within normal ranges) at weeks 4, 8, and 12 after the first dose administration. 4) Changes in the end-stage liver disease model (MELD) score and the Asian Association for Research on Liver Diseases ACLF Research Consortium (AARC) score of ACLF patients at weeks 4, 8, and 12 after the first administration compared to baseline. 5) The incidence of switching to other treatment modalities (artificial liver, liver transplantation, cell therapy, etc.). 6) Liver transplantation rate on day 90; 7) Pharmacokinetic parameters of AS1501 after multiple administrations, including steady-state plasma minimum concentration (C_{ss-min}). 8) Immunogenicity-related indicators, such as ADA antibodies. 9) The incidence rates of various adverse events (AEs) and serious adverse events (SAEs), as well as the incidence rates of AEs and SAEs associated with the study product. 10) Preliminary investigation into the changes in serum TRAIL and NK cell
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	membrane-type TRAIL expression levels before and after administration of AS1501 for injection in ACLF patients. Exploratory endpoints: 1) Preliminary investigation into cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.
Study population and sample size	Stage IIa: Intended to include patients with early-stage ACLF, with two dose groups of 0.5 mg/kg and 1 mg/kg planned, each comprising 12 subjects for a total of 24 subjects. Phase IIb: Subjects with ACLF (early or intermediate stages) were planned to be enrolled and randomly assigned in a 1:1 ratio to receive either the appropriate dose of the investigational drug or placebo. Each group was intended to enroll 36 subjects, totaling 72 subjects.
research design	This trial is a Phase II clinical study conducted in two phases: Phase IIa: A sentinel, single-arm study design is planned to enroll 12 early-stage ACLF patients receiving the 0.5 mg/kg dose regimen. The first two participants will serve as sentinels, enrolled sequentially and receiving a single intravenous dose. If these two sentinels do not experience any study drug-related serious adverse events (SAEs) within 2 weeks after the initial dose, the remaining 10 participants may be enrolled; otherwise, the dose will be reduced and the exploration phase continued. After the single intravenous dose, participants must undergo an 20-day washout period. During this washout period, if no study drug-related adverse events of grade ≥ 3 occur and safety and tolerability are jointly assessed by investigators and the sponsor, the participant proceeds to the multiple-dose phase (once weekly [with D21 being the first dose of multiple administration], for 4 consecutive weeks); otherwise, the dose will be reduced and the exploration phase continues, with the specific dose determined through joint discussion between the sponsor and investigators. If any participant drops out during the washout period after the single dose, additional participants may be recruited to ensure at least 12 subjects enter the multiple-dose phase. After all 12 early-stage ACLF subjects in the 0.5 mg/kg dose group completed continuous dosing, the Safety Monitoring Committee (SMC) evaluated the safety profile of this dose group. The SMC would discuss whether to proceed with dose escalation if any of the following conditions occurred in the 0.5 mg/kg group: 1) At least one-third of the subjects experienced drug-related grade 3 adverse events (AEs). 2) Any drug-related adverse event (AE) of grade 4 or higher. If, upon SMC evaluation, the subjects meet the dose escalation criteria, the remaining 12 early-stage ACLF participants eligible for enrollment will receive the 1 mg/kg dose group. Following the same enrollment rules as the 0.5 mg/kg dose group, the first two participants serve as sentinels and are enrolled sequentially for a single intravenous

	administration. If these two sentinels do not experience any study drug-related serious adverse events (SAEs) within 2 weeks after the initial dose, the remaining 10 participants may be enrolled; otherwise, the dose will be reduced to continue the exploratory study. After the single intravenous dose, subjects must undergo an 20-day washout period. During this washout period, if no study drug-related adverse events of grade ≥ 3 occur and both the investigator and the sponsor jointly assess safety and tolerability, the subject enters the multiple-dose phase (once weekly [with D21 being the first dose of multiple administration], for 4 consecutive weeks). If any participant drops out during the washout period after the single dose, additional subjects may be recruited to ensure that at least 12 participants enter the multiple-dose phase. Following completion of dose exploration studies at 0.5 mg/kg and 1 mg/kg, investigators and the sponsor may determine the recommended appropriate dose for Phase IIb trials based on cumulative safety data, efficacy results, and potential PK/PD findings; alternatively, they may explore other dose ranges or dosing frequencies. Phase IIb: A randomized (1:1), double-blind, placebo-controlled parallel-design study was planned to enroll 72 ACLF patients receiving either appropriate doses and frequencies of AS1501 or placebo, with the aim of further evaluating the efficacy and safety of AS1501 for injection. The specific study design will be determined jointly by the investigators and the sponsor based on the results from Phase IIa. The research process at each stage of this study is divided into the screening phase, treatment phase, and safety follow-up phase. The specific design is shown in the figure below. IIa期，患者单次、多次给药剂量递增，单臂，开放 适应症：ACLF早期 N=12 (前2例为哨兵)★ ★剂量递增 洗脱期 (20天) 多次给药，每周一次，共4次 1mg/kg 首次 1mg/kg 1mg/kg 1mg/kg 1mg/kg N=12 (前2例为哨兵)★ 洗脱期 (20天) 多次给药，每周一次，共4次 0.5mg/kg 首次 0.5mg/kg 0.5mg/kg 0.5mg/kg 0.5mg/kg ★哨兵未发生与研究药物相关的SAE；若出现与研究药物相关的SAE，则评估是否降低剂量组 ★未发生任何与研究药物相关的≥ 3级AE ★未发生：$\geq 1/3$受试者出现药物相关3级的AE；任何4级和4级以上的药物相关的AE IIb期，患者多次给药，随机、双盲、安慰剂对照、探索疗效和安全性 适应症：ACLF (慢加急性肝功能衰竭) 早期中期 ACLF 筛选 随机 1:1 研究药物，合适剂量，多次给药，N=36 安慰剂，合适剂量，多次给药，N=36 安全性随访 90天
Inclusion and Exclusion Criteria	➤ Stage IIa – Main inclusion criteria (1) The age of the signatories to the informed consent form must range from 18 to 75 years old (including the boundary values). (2) During screening, chronic plus acute liver failure was diagnosed based on the Guidelines for Diagnosis and Treatment of Liver Failure (2018 Edition) issued by the Liver Failure and Artificial Liver Group of the Infectious Diseases Branch of

	the Chinese Medical Association and the Severe Liver Disease and Artificial Liver Group of the Hepatology Branch of the Chinese Medical Association. Patients were in the early stage of acute liver failure (ACLF) and did not meet the criteria for liver transplantation. ◆ Early manifestations of ACLF: • Severe fatigue accompanied by significant gastrointestinal symptoms such as marked anorexia, vomiting, and abdominal distension; • ALT and/or AST levels remain significantly elevated, with progressive worsening of jaundice (TBil $\geq 171 \mu\text{mol/L}$ or daily increase $\geq 17.1 \mu\text{mol/L}$); • Hemorrhagic tendency is present in 30% of cases with PTA values ranging from $<30\%$ to $\leq 40\%$ (or INR values between 1.5 and <1.9). • No complications or other extrahepatic organ failures were observed. (3) During the screening phase, serum TRAIL levels are elevated to ≥ 2 times that of normal individuals, or NK cell membrane-type TRAIL expression is increased. (4) Can comprehend the informed consent form, voluntarily participate in it, and sign it; (5) Capable of completing the trial in accordance with the study protocol; (6) Participants (including their partners) are willing to voluntarily adopt effective contraceptive measures from the screening stage through 6 months after the last administration of the investigational drug. ➤ Stage IIa – Main exclusion criteria (1) Patients with a history of allergic predisposition or severe hypersensitivity to protein-based drugs (CTCAE v5.0 grade>3); (2) Patients who have undergone liver transplantation or plan to undergo liver transplantation within 1 month; (3) ACLF patients at intermediate or advanced stages; (4) Severe Grade III ascites or refractory ascites; (5) Associated with stage III-IV hepatic encephalopathy; (6) Patients with concurrent malignant tumors or a history of malignant tumors; those diagnosed with lung cancer, liver cancer, pancreatic cancer, or gastrointestinal tumors through imaging studies (ultrasound, CT, or MRI) and tumor marker tests (e.g., AFP, CEA, CA125, or CA199) during the screening period or within one month prior to it.
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	(7) Patients during the screening period or within 1 month prior to screening whose endoscopic or imaging (abdominal ultrasound, CT, or MRI) findings indicate severe varices with a bleeding risk; (8) The KDIGO criteria for acute kidney injury (AKI) define the following subjects: (1) a serum creatinine (Scr) level increase of $\geq 26.5 \mu\text{mol/L}$ (0.3 mg/dL; $1 \text{ mg/dL} = 88.4 \mu\text{mol/L}$) within 48 hours; (2) a Scr increase of ≥ 1.5 times the baseline value within 7 days; and (3) reduced urine output ($<0.5 \text{ ml/kg/h}$) lasting for more than 6 hours. (9) The following laboratory test values are present or abnormal: a. Complete blood count: platelets (PLT) $<75 \times 10^9/\text{L}$, hemoglobin (HGB) $<80 \text{ g/L}$; b. PT-INR ≥ 1.9 or PTA $\leq 30\%$; c. Left ventricular ejection fraction (LVEF) $<50\%$; serum creatinine $>1.5 \times \text{ULN}$. (10) Those with severe respiratory dysfunction, dyspnea, or respiratory failure; (11) Severe infections that cannot be controlled by concomitant medications, including infections of major organs such as the abdominal cavity, lungs, urinary tract, and skin; (12) Individuals who are HIV-positive, or have active pulmonary tuberculosis or syphilis infection; (13) Patients with a history of unstable ischemic heart disease, congestive heart failure, myocardial infarction (MI), stroke, severe arrhythmias, or other related conditions; (14) Patients with severe hypertension or diabetes that cannot be controlled with concomitant medications; (15) Women who are pregnant or breastfeeding, or those with a positive pregnancy test result; (16) Individuals who participated in clinical trials involving other drugs or medical devices within 30 days prior to randomization or during any of the five drug half-life periods; (17) The study subjects must have undergone trauma or major surgery (e.g., requiring general anesthesia) within 28 days prior to the first administration of the investigational drug. Note: Subjects scheduled for surgical procedures under local anesthesia are eligible to participate in the study. (18) Any serious potential medical or psychiatric condition (e.g., alcohol or drug abuse), dementia, or changes in mental status; or any issue that may impair the subject's ability to receive or tolerate the planned treatment at the research center, understand informed consent, or participate in the study, or that investigators consider contraindications for enrollment or potential interference
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	with protocol-defined assessments or study outcomes. (19) The researchers identified other conditions that made participants unsuitable for this study. ➤ Stage IIb – Main inclusion criteria Individuals who do not meet any of the following criteria shall not be enrolled as subjects. (1) The age of the signatories to the informed consent form must range from 18 to 75 years old (including the boundary values). (2) The screening criteria were based on the "Guidelines for Diagnosis and Treatment of Liver Failure (2018 Edition)" issued by the Liver Failure and Artificial Liver Group of the Infectious Diseases Branch of the Chinese Medical Association and the Severe Liver Disease and Artificial Liver Group of the Hepatology Branch of the Chinese Medical Association, requiring a diagnosis of chronic plus acute liver failure at an early or intermediate stage of ACLF (Acute Complicated Liver Failure) and failure to meet the eligibility criteria for liver transplantation. ◆ Early manifestations of liver failure: • Severe fatigue accompanied by significant gastrointestinal symptoms such as marked anorexia, vomiting, and abdominal distension; • ALT and/or AST levels remain significantly elevated, with progressive worsening of jaundice (TBil $\geq 171 \mu\text{mol/L}$ or daily increase $\geq 17.1 \mu\text{mol/L}$); • Hemorrhagic tendency is present in 30% of cases with PTA values ranging from $<30\%$ to $\leq 40\%$ (or INR values between 1.5 and <1.9). • No complications or other extrahepatic organ failures were observed. ◆ Mid-stage manifestations of liver failure: • Based on the early manifestations of liver failure, the condition further progresses. • ALT and/or AST levels decline rapidly, while TBil continues to rise. • The bleeding is evident (with petechiae or ecchymoses), with $20\% < \text{PTA} \leq 30\%$ (or $1.9 \leq \text{INR} < 2.6$). • Associated with one complication and/or one extrahepatic organ failure.
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	(3) During the screening phase, serum TRAIL levels are elevated to ≥ 2 times that of normal individuals, or NK cell membrane-type TRAIL expression is increased. (4) Can comprehend the informed consent form, voluntarily participate in it, and sign it; (5) Can comply with the study protocol and complete the trial. (6) Participants (including their partners) are willing to voluntarily adopt effective contraceptive measures from the screening stage through 6 months after the last administration of the investigational drug. ➤ Stage IIb – Main exclusion criteria Subjects are ineligible for inclusion in this study if any of the following criteria are met. (1) Patients with a history of allergic predisposition or severe hypersensitivity to protein-based drugs (CTCAE v5.0 grade>3); (2) Patients who have undergone liver transplantation or plan to undergo liver transplantation within 1 month; (3) Patients with advanced ACLF; (4) Severe Grade III ascites or refractory ascites; (5) Associated with stage III-IV hepatic encephalopathy; (6) Patients with concurrent malignant tumors or a history of malignant tumors; those diagnosed with lung cancer, liver cancer, pancreatic cancer, or gastrointestinal tumors through imaging studies (ultrasound, CT, or MRI) and tumor marker tests (e.g., AFP, CEA, CA125, or CA199) during the screening period or within one month prior to it. (7) Patients during the screening period or within 1 month prior to screening whose endoscopic or imaging (abdominal ultrasound, CT, or MRI) findings indicate severe varices with a bleeding risk; (8) Patients with severe coagulation failure defined as PT-INR ≥ 2.6 or PTA $\leq 20\%$; complete blood count: platelets (PLT) $<75 \times 10^9/L$, hemoglobin (HGB) $<80 \text{ g/L}$; (9) Those with severe respiratory dysfunction, dyspnea, or respiratory failure; (10) Severe infections that cannot be controlled by concomitant medications, including infections of major organs such as the abdominal cavity, lungs, urinary tract, and skin; (11) Individuals who are HIV-positive, or have active pulmonary tuberculosis or syphilis infection; (12) Patients with a history of unstable ischemic heart disease, congestive heart failure, myocardial infarction (MI), stroke, severe arrhythmias, or other related conditions;
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	(13) Patients with severe hypertension or diabetes that cannot be controlled with concomitant medications; (14) Women who are pregnant or breastfeeding, or those with a positive pregnancy test result; (15) Individuals who participated in clinical trials involving other drugs or medical devices within 30 days prior to randomization or during any of the five drug half-life periods; (16) The study subjects must have undergone trauma or major surgery (e.g., requiring general anesthesia) within 28 days prior to the first administration of the investigational drug. Note: Subjects scheduled for surgical procedures under local anesthesia are eligible to participate in the study. (17) Any serious potential medical or psychiatric condition (e.g., alcohol or drug abuse), dementia, or changes in mental status; or any issue that may impair the subject's ability to receive or tolerate the planned treatment at the research center, understand informed consent, or participate in the study, or that investigators consider contraindications for enrollment or potential interference with protocol-defined assessments or study outcomes. (18) The researchers identified other conditions that made participants unsuitable for this study.
research on drug	Test drug: AS1501 for Injection, dosage form: lyophilized powder for injection; specification: 25 mg/bottle; characteristics: white loose powder; batch number: ****; expiration date: ** year ** month ** day; storage conditions: sealed, protected from light, refrigerated at 2°C–8°C; supplied by Shenzhen Zhongke Aishen Pharmaceutical Co., Ltd. Basic therapeutic agents: ACLF patients may receive targeted treatment or management based on the underlying etiology and precipitating factors. These include, but are not limited to, necessary nutritional support, plasma transfusion (excluding within 48 hours prior to AARC or MELD score assessment), albumin supplementation, intravenous catheterization, dynamic monitoring, fluid resuscitation, microbiota modulation, analgesics, sedatives, antibiotics, oxygen therapy, and mechanical ventilation. The use of artificial liver support, glucocorticoids (excluding pre-treatment regimens or treatments for infusion-related adverse events/cytokine release syndrome), and hepatocyte growth factor during the trial is prohibited.
Standards for Terminating Treatment	Subjects may request discontinuation of treatment at any time, and all reasons must be recorded in the Case Report Form (CRF), including but not limited to the following: • The subject withdrew their informed consent. • Unbearable toxicity occurs;

	• Poor compliance; • The subject requests to terminate treatment (e.g., considering consideration of artificial liver support); • Lost to follow-up; • die ; • encyesis ; • other ;
Exit Study Criteria	Subjects may request to terminate or withdraw from the study at any time, and all reasons must be recorded in the Case Report Form (CRF), including but not limited to the following: <u>The subject voluntarily withdrew from the study.</u> • The subject withdrew their informed consent. • encyesis ; • Lost to follow-up; • die ; • other ; <u>The subjects selected by the researchers were discontinued or withdrawn from the study.</u> • Significant protocol deviations or violations occurred during the study, which substantially impacted drug tolerance, safety, or pharmacokinetic (PK) evaluation. • From a medical ethics perspective, the researchers deemed it necessary to terminate the trial for patients requiring urgent liver transplantation who have suitable donor liver sources available. • Patients who experienced life-threatening serious adverse events (SAEs) related to the investigational drug and are not suitable for continuing participation in the trial; • After discontinuing the study drug therapy, the researchers determined that withdrawal from the study was most beneficial for the subject. • Poor subject compliance (e.g., failure to adhere to prescribed medication regimens, examination protocols, or use of other drugs that may affect efficacy and safety evaluation outcomes); severe protocol violations/deviations; • The occurrence of a clinical adverse event (AE), laboratory abnormalities, or other medical conditions may pose a risk to the subject's safety or health if continued participation in the study is warranted, and would not maximize the subject's benefit.

	Subjects meeting the exclusion criteria (newly identified or previously undetected) are not eligible to continue participating in the study.
Study Conclusion Definition	Stage IIa: The last participant completed the final follow-up as specified in the protocol. Phase IIb: The last participant completes a 4-week efficacy and safety assessment or withdraws from the study early (whichever occurs first), and long-term follow-up data can be continuously collected.
dosage regimen	Administration method: Administer by intravenous infusion. Reconstitute AS1501 for injection with sterile water for injection, then add 0.9% sodium chloride injection (the total volume of the test drug, sterile water for injection, and 0.9% sodium chloride injection should be 200 mL). The infusion duration should not be less than 2 hours. During Phase IIb, the administration method for placebo was identical to that of AS1501. Pre-treatment before AS1501 administration: In the Phase I clinical study of AS1501 in healthy volunteers, two cases of grade 3 hypersensitivity reactions occurred in the 2 mg/kg dose group, and two cases of cytokine release syndrome (CRS) were observed in the 3 mg/kg dose group. Therefore, in this study, if any dose group in Phase IIa exhibits $\geq$ grade 2 hypersensitivity reactions or infusion reactions related to the investigational drug, or if CRS of any severity is observed, the affected subject as well as other subjects enrolled subsequently may receive pre-treatment prior to investigational drug administration. This treatment should include at least glucocorticoids (dexamethasone 5 – 10 mg or equivalent agents), supplemented by antihistamines (oral or intravenous diphenhydramine 25 mg–50 mg) and antipyretic analgesics (oral acetaminophen 650 mg – 1000 mg) as per institutional protocols. Administration frequency: Initially scheduled once every 7 days; for Phase IIa, five doses are planned (one initial single dose followed by four consecutive doses). For Phase IIb, once weekly for a total of four doses. The specific dosing regimen (including number of doses and frequency) will be determined based on Phase IIa data.
cell factor	Stage IIa sampling time points: Before each infusion (within 0.5 h $\pm$ 5 min) on days D1, D21, D28, D35, and D42; and at 0 h ($\pm$ 30 min) and 24 h ($\pm$ 30 min) after each infusion completion for sample collection. For Phase IIb, sample collection was performed at the following time points: before each infusion (within 0.5 h $\pm$ 5 min) on days D1/D8/D15/D22; at 0 h ($\pm$ 30 min) and 24 h ($\pm$ 30 min) after each infusion completion.
PK draw	Stage IIa: Blood samples were collected at the following time points: before

blood	infusion initiation on D1 (within 0.5 h $\pm$ 5 min), 1 h $\pm$ 5 min after infusion start on D1, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, and 24 h $\pm$ 30 min after infusion completion (D2); 72 h $\pm$ 30 min (D4); 144 h $\pm$ 30 min (D7); 312 h $\pm$ 30 min (D14); prior to dosing on D21/D28/D35 (within 0.5 h $\pm$ 5 min); prior to dosing on D42 (within 0.5 h $\pm$ 5 min); and again at 1 h $\pm$ 5 min after infusion start, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, and 24 h $\pm$ 30 min after infusion completion (D43); 72 h $\pm$ 30 min (D45); 144 h $\pm$ 30 min (D48); and 312 h $\pm$ 30 min (D55). A total of 23 PK blood sampling time points were tentatively designated. Phase IIb: Subjects underwent collection of plasma samples at trough concentration PK measurements before infusion on days D8, D15, and D22 (within 0.5 h $\pm$ 5 min), and at peak concentration PK measurements immediately after infusion completion on days D1 and D22 (at 0 h $\pm$ 5 min). A total of five PK sampling time points were provisionally designated. During the trial, the subsequent PK blood sampling sites may be adjusted based on preliminary PK test results.
immunogenicity	Stage IIa: Before drug administration on Day 1 (within 0.5 h $\pm$ 5 min), at 144 h $\pm$ 30 min post-administration on Day 1 (Day 7), at 312 h $\pm$ 30 min post-administration on Day 14, and before drug administration on Days 21,28,35, and 42 (within 0.5 h $\pm$ 5 min); immunogenicity blood samples were collected at each of these time points during the final visit, with a total of eight sampling time points designated. Stage IIb: Immunoactive blood samples were collected before administration of D1/D8/D15/D22 (within 0.5 h $\pm$ 5 min), totaling four time points. During the trial, the subsequent design of immunogenicity blood sampling sites may be adjusted based on preliminary immunogenicity test results.
TRAIL Sample Collection	Stage IIa: Screening phase, with blood sampling at 12 PD time points: before D1 infusion (within 0.5 h $\pm$ 5 min), at 0 h $\pm$ 5 min after D1 infusion completion, at 144 h $\pm$ 30 min (D7), at 312 h $\pm$ 30 min (D14), before administration on D21/D28/D35 (within 0.5 h $\pm$ 5 min), before D42 infusion (fourth consecutive administration) (within 0.5 h $\pm$ 5 min), and at 0 h $\pm$ 5 min and 144 h $\pm$ 30 min after D42 infusion completion (D48), as well as at 312 h $\pm$ 30 min after D55 infusion. Stage IIb: Blood samples for TRAIL analysis were collected at six designated

	time points during the screening phase, prior to administration of D1/D8/D15/D22 (within 0.5 h ± 5 min), and at the end-of-treatment visit. During the trial, the subsequent PD blood collection site design may be adjusted based on preliminary results.
Biological Sample Analysis Methods	The concentration of AS1501 in serum will be determined using validated methods such as enzyme-linked immunosorbent assay (ELISA) or microtiter plate-based chemiluminescence assay (MSD). The proposed method involves using validated techniques such as ELISA or MSD to detect ADA against AS1501 in serum. The validated ELISA method will be employed to determine the concentration levels of TRAIL in serum. Flow cytometry will be employed to determine the expression levels of TRAIL on NK cell membranes.
safety evaluation	Safety Monitoring: During the trial, subjects were closely monitored for physical examination, vital signs, electrocardiogram (ECG), complete blood count (CBC), urinalysis, blood biochemistry parameters, coagulation function, and adverse events.
statistical method	Statistical analysis will be performed using SAS version 9.4 or later. Descriptive analysis will be conducted for safety and efficacy data. Adverse events will be coded using MedDRA (International Medical Terminology Dictionary). Analysis of adverse events will be based on events occurring after administration (TEAEs). The number of subjects experiencing adverse events and their incidence rates will be calculated. Descriptive statistics will be employed to summarize vital signs and laboratory test results. A shift table will be used to describe changes in normal/abnormal laboratory test results before and after administration. <u>Stage IIa analysis population</u> Subjects will be analyzed using the following analytical datasets: • Safety Analysis Set: All subjects who received at least one dose (or any portion thereof) of the study drug (AS1501) and had recorded safety parameters. This analysis set will be used for all safety analyses, with subjects analyzed based on their actual treatment regimen. • Full Analysis Set: All subjects who received at least one dose of the study drug (AS1501). This analysis set will be used for all efficacy analyses. • Pharmacokinetic analysis cohort: All subjects who received at least one dose of AS1501, completed blood sampling at ≥60% (3/5) of the first consecutive dosing points, did not experience protocol deviations that

	significantly affected PK parameters (e.g., Cmax, AUC), and had at least one evaluable PK parameter. This cohort will be used for all pharmacokinetic analyses, with subjects analyzed based on their actual treatment regimen. • Pharmacokinetic analysis cohort: All subjects who received at least one dose of the study drug and had at least one post-treatment pharmacokinetic (biomarker) measurement. Subjects will be analyzed based on their treatment regimen. • Immunogenicity Analysis Cohort: All subjects who received at least one dose of the study drug and had at least one post-treatment immunogenicity measurement. This cohort will be used for immunogenicity analysis, with subjects analyzed based on their actual treatment regimen. <u>Phase IIb analysis population</u> Subjects will be analyzed using the following analytical datasets: • Safety Analysis Set: All subjects who received at least one dose (or any portion thereof) of the study drug (AS1501 or placebo) and had recorded safety parameters. This analysis set will be used for all safety analyses, with subjects analyzed based on their actual treatment regimen. • Full Analysis Set: All randomized subjects who received at least one dose of the study drug (AS1501 or placebo). This analysis set will be used for all efficacy analyses, and subjects will be analyzed according to their assigned treatment group. • Eligible analysis cohort: All randomized subjects who met the inclusion criteria, did not meet the exclusion criteria, completed at least 3 doses (75%) of the study drug (AS1501), and experienced no major protocol deviations affecting efficacy evaluation. This analysis cohort will be used to supplement the efficacy analysis based on FAS. • Pharmacokinetic analysis cohort: All subjects who received at least one dose of the study drug (AS1501) and had at least one evaluable plasma concentration measurement. This cohort will be used for all pharmacokinetic analyses, with subjects analyzed based on their actual treatment regimen. • Pharmacokinetic analysis cohort: All randomized subjects who received at
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	least one dose of the study drug (AS1501 or placebo) and had at least one post-treatment pharmacokinetic (biomarker) measurement. Subjects will be analyzed according to their assigned treatment group. • Immunoorigidity analysis cohort: All subjects who received at least one dose of the study drug (AS1501 or placebo) and had at least one post-treatment immunoorigidity measurement. This cohort will be used for immunoorigidity analysis, with subjects analyzed according to their randomized treatment assignment.
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		stage of therapy									follow-up period	
Visit Description	Screening Period	Single administration period + Elution period after single administration (D1–20)					Multiple dosing periods (D21–D42)				End Treatment/Exit Study	Safety Follow-up
Visit Date (Days)	D-14~1	D1	D2	D4	D7	D14	D21	D28	D35	D42	Within 14 days after the last administration	28 days after the last administration
Time Window (± days)	-	-	±1	±1	±1	±2	±2	±2	±2	±2	±3	±7
Sign the Informed Consent Form 1	×											
Demographic Statistics Data 2	×											
Previous medical history and treatment history, personal history 3	×											
Selection and Exclusion Criteria 4	×											
Life Signs 5	×	×			×	×	×	×	×	×	×	
Physical Examination 6	×	×					×	×	×	×	×	
Height, Weight, BMI 7	×	×					×	×	×	×	×	
Blood pregnancy test (for women of childbearing age) 8	×	×									×	
Virus Screening 9	×											
Abdominal CT (digestive system) 10	×											
echocardiogram	×											
12-lead electrocardiogram 11	×	×			×	×	×	×	×	×	×	

		stage of therapy									follow-up period	
Visit Description	Screening Period	Single administration period + Elution period after single administration (D1–20)					Multiple dosing periods (D21–D42)				End Treatment/Exit Study	Safety Follow-up
Visit Date (Days)	D-14~1	D1	D2	D4	D7	D14	D21	D28	D35	D42	Within 14 days after the last administration	28 days after the last administration
Time Window (± days)	-	-	±1	±1	±1	±2	±2	±2	±2	±2	±3	±7
Complete Blood Count 12	×	×			×	×	×	×	×	×	×	
Blood Biochemistry 13	×	×			×	×	×	×	×	×	×	
Coagulation Function 14	×	×			×	×	×	×	×	×	×	
Urinalysis result 15	×	×			×	×	×	×	×	×	×	
Arterial blood gas analysis 16	×	×					×	×	×	×	×	
Inflammatory markers (CRP/procalcitonin): 17	×	×									×	
Child-Pugh score: 18	×	×					×	×	×	×	×	
ACLF Research Alliance (AARC) scoring system developed by the Asia-Pacific Liver Disease Research Association	×	×					×	×	×	×	×	
MELD score for end-stage liver disease: 20	×	×					×	×	×	×	×	
Diagnosis of SIRS 21	×	×					×	×	×	×	×	
AS1501 Administration 22		×					×	×	×	×		
Cytokine 23		×	×				×	×	×	×		

		stage of therapy									follow-up period	
Visit Description	Screening Period	Single administration period + Elution period after single administration (D1–20)					Multiple dosing periods (D21–D42)				End Treatment/Exit Study	Safety Follow-up
Visit Date (Days)	D-14~1	D1	D2	D4	D7	D14	D21	D28	D35	D42	Within 14 days after the last administration	28 days after the last administration
Time Window (± days)	-	-	±1	±1	±1	±2	±2	±2	±2	±2	±3	±7
Immunogenicity 24		×			×	×	×	×	×	×	×	
Pharmacokinetics (PK) 25		×	×	×	×	×	×	×	×	×		
Cytokine release induced by AS1501 29		×	×	×	×	×	×	×	×	×		
AS1501 In vivo receptor saturation		×	×	×	×	×	×	×	×	×		
PD biomarker (TRAIL) 26	×	×			×	×	×	×	×	×		
Event Record 27: Adverse Event	×											
Combined Medications 28	×											

- Participants must sign an informed consent form prior to undergoing any study-related procedures.
- Demographic data include age, gender, and ethnicity.
- Medical and treatment history: Includes the disease history of previous liver disorders, as well as the medical history and treatment history of other conditions; the history of liver diseases should specify the exact diagnosis, diagnostic date, and previously administered therapeutic interventions. The subject's history of other diseases and treatments should be obtained and documented. Personal history encompasses smoking history, alcohol consumption history, surgical or traumatic history, allergy history, infectious disease history, and drug abuse/treatment history.
- Eligibility criteria for subjects are determined during the screening phase.
- The vital sign monitoring items include body temperature, respiration, pulse/heart rate, and blood pressure; measurements should be taken after 10 minutes of rest. Time points for monitoring are: screening period (within 3 days prior to initial administration), Day 1 (within 0.5 h ± 10 min before administration, at 1 h ± 10 min, 2 h ± 10 min, 4 h ± 20 min after infusion initiation, Days 7, 14, 21, 28, 35, and 42 [within 0.5 h ± 10 min before administration, at 1 h ± 10 min, 2 h ± 10 min, 4 h ± 20 min after infusion initiation], and the treatment conclusion visit.

- 6 The comprehensive physical examination covers general condition, head and neck, lymph nodes, chest, abdomen, musculoskeletal system, skin, nervous system, and other areas, and will be conducted during the screening visit. During subsequent visits, a simplified examination will be performed, including but not limited to general assessment and any abnormal signs deemed significant by the investigator.
- 7 Height and BMI were collected solely during the screening period. Weight measurements were taken at the following time points: screening period, before administration on Day 1, before administrations on Days 21/28/35/42, and at the treatment completion visit.
- 8 Only women of childbearing age require blood pregnancy testing; testing time points include the screening period, before administration on Day 1 (results within 3 days prior to the first dose are acceptable), and at the end-of-treatment visit. Researchers may perform additional serum pregnancy tests based on clinical indications.
- 9 Viral screening includes: hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), hepatitis C antibody (Anti-HCV), hepatitis B virus DNA (HBV-DNA), and hepatitis C virus RNA (HCV-RNA). HBV-DNA testing should be performed when HBsAg is positive or when deemed necessary by the investigator, while HCV-RNA testing should be conducted when required by the investigator. Additionally, human immunodeficiency virus antibody (HIV) and Treponema pallidum antibody tests are included. The testing time point is during the screening phase. For patients with chronic hepatitis virus infection, regular follow-up monitoring of viral-related indicators may be recommended by the investigator.
- 10 Abdominal CT (digestive system): Abdominal CT scans must be completed during the screening period. High-quality results obtained within one month from any medical center are acceptable.
- 11 12-lead electrocardiogram (ECG): Includes parameters such as heart rate, RR interval, PR interval, QT interval, QRS interval, and QTc interval. Measurement time points are as follows: screening period (within 14 days prior to initial administration), within 0.5 h $\pm$ 10 min before D1 administration, at 0 h $\pm$ 10 min, 1 h $\pm$ 10 min, 2 h $\pm$ 10 min, and 8 h $\pm$ 20 min after infusion completion on D1, on D7, D14, and before administrations on D21/D28/D35/D42 (within 0.5 h $\pm$ 10 min), at 0 h $\pm$ 10 min, 1 h $\pm$ 10 min, 2 h $\pm$ 10 min, and 8 h $\pm$ 20 min after infusion completion on D21/D28/D35/D42, and during the final treatment visit. If abnormalities such as prolonged QTcF interval occur in subjects, investigators may increase the frequency of examinations based on individual circumstances; post-administration, ECG monitoring may be added upon investigator assessment. Prior to each examination, subjects should rest for at least 10 minutes. If blood sampling is scheduled on the same day, ECG monitoring must be completed within 30 minutes prior to sampling.
- 12 The complete blood count (CBC) parameters include: white blood cell count (WBC), absolute neutrophil count (ANC), neutrophil percentage, eosinophil percentage, basophil percentage, lymphocyte count (LYM), lymphocyte percentage, monocyte count (MONO), monocyte percentage, red blood cell count (RBC), hemoglobin (HGB), and platelet count (PLT). Measurement time points are: screening period; before day 1 of administration (results within 3 days prior to first dose are acceptable); day 7 (results within 1 day prior are acceptable); day 14 (results within 1 day prior are acceptable); before days 21,28,35, or 42 of treatment (results within 1 day prior are acceptable); and at the end-of-treatment visit. During the treatment phase, additional tests may be performed by the investigator based on the subject's condition.
- 13 Blood biochemical test items: including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin (ALB), pre-albumin (Pre-ALB), total bilirubin (TBIL), direct bilirubin (DBIL), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatinine (CREA), urea (UREA), uric acid (UA), potassium (K), sodium (Na), chloride (Cl), calcium (Ca), magnesium (Mg), phosphorus (P), glucose, lactate dehydrogenase (LDH), and creatine kinase. Testing time points: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); Day 7 (results within 1 day prior are acceptable); Day 14 (results within 1 day prior are acceptable); before administration on Days 21,28,35,

- or 42 (results within 1 day prior are acceptable); and at the treatment conclusion visit. During the treatment period, additional tests may be performed by the investigator based on the subject's condition.
- 14 Coagulation function tests include: prothrombin time (PT), activated partial thromboplastin time (APTT), international normalized ratio (INR), fibrinogen (FIB), and prothrombin activity (PTA). Testing points are specified as follows: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); Day 7 (results within 1 day prior are acceptable); Day 14 (results within 1 day prior are acceptable); before administrations on Days 21,28,35, or 42 (results within 1 day prior are acceptable); and at the end-of-treatment visit. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 15 Urinalysis parameters include: pH, glucose, protein, occult blood, ketones, bilirubin, urobilinogen, nitrite, white blood cells, red blood cells, and specific gravity. Testing time points are: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); Day 7 (results within 1 day prior are acceptable); Day 14 (results within 1 day prior are acceptable); before administrations on Days 21,28,35, or 42 (results within 1 day prior are acceptable); and at the treatment conclusion visit. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 16 Arterial blood gas analysis includes the following parameters: pH, partial pressure of oxygen (PO_2), arterial oxygen saturation (SaO_2), partial pressure of carbon dioxide (PCO_2), and lactate levels. Measurement time points are specified as follows: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); before administrations on Days 21,28,35, and 42 (results within 1 day prior to administration are acceptable); and at the end-of-treatment visit. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 17 Inflammatory markers: including C-reactive protein (CRP), procalcitonin, etc.; Testing time points: screening period, before administration on Day 1 (results within 3 days prior to the first dose are acceptable), and at the end-of-treatment visit. During the treatment period, additional tests may be performed by the investigator based on the subject's condition.
- 18 Child-Pugh score: During the screening phase, liver function should be assessed using the modified Child-Pugh classification system. Reference parameters include hepatic encephalopathy, ascites, total bilirubin, albumin levels, and prolonged prothrombin time. Based on these parameters, the scores are categorized as: Grade A (5–6 points), Grade B (7–9 points), or Grade C (≥ 10 points).
- 19 ACLF Research Alliance (AARC) scoring system developed by the Asia-Pacific Liver Disease Research Association. Time points: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); before administrations on Days 21,28,35, and 42; and at the end-of-treatment visit.
- 20 MELD score for end-stage liver disease. Time points: screening period; before administration on Day 1 (results within 3 days prior to first dose are acceptable); before administrations on Days 21,28,35, and 42; and at the treatment conclusion visit.
- 21 SRIS: Includes body temperature, heart rate, respiratory rate, and white blood cell count. Time points: screening period; before administration on Day 1 (results within 3 days prior to the first dose are acceptable); before administrations on Days 21,28,35, and 42; and at the end-of-treatment visit.
- 22 AS1501 administration.

23 Cytokines: At least TNF- α and IL-6 must be included; investigators may add additional cytokine assays based on the available testing panels at each center and the subject's condition.

Sampling time points: samples shall be collected before each infusion (within 0.5 h $\pm$ 5 min) on days D1, D21, D28, D35, and D42; at 0 h ($\pm$ 30 min) and 24 h ($\pm$ 30 min) after each infusion on days D1, D21, D28, D35, and D42. During the treatment period, additional tests may be performed by the investigator based on the subject's clinical status.

24 Immunogenicity: Immune response samples were collected at eight designated time points, including before D1 administration (within 0.5 h $\pm$ 5 min), 144 h $\pm$ 30 min after D1 administration (D7), 312 h $\pm$ 30 min after D1 administration (D14), and before D21/D28/D35/D42 administrations (within 0.5 h $\pm$ 5 min) as well as during the final treatment visit.

25 Pharmacokinetics (PK): Blood samples were collected at the following time points: before D1 infusion initiation (within 0.5 h $\pm$ 5 min), 1 h $\pm$ 5 min after D1 infusion start, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, 24 h $\pm$ 30 min (D2), 72 h $\pm$ 30 min (D4), 144 h $\pm$ 30 min (D7), 312 h $\pm$ 30 min (D14); pre-administration of D21/D28/D35 (within 0.5 h $\pm$ 5 min); pre-administration of D42 (within 0.5 h $\pm$ 5 min); and post-administration at 1 h $\pm$ 5 min, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, 24 h $\pm$ 30 min (D43), 72 h $\pm$ 30 min (D45), 144 h $\pm$ 30 min (D48), 312 h $\pm$ 30 min (D55). A total of 23 PK sampling time points were designated.

26 TRAIL: This test primarily measures serum TRAIL levels and membrane-bound TRAIL levels on NK cells. Samples should be collected at 12 PD time points: during the screening phase; before D1 infusion (within 0.5 h $\pm$ 5 min); at 0h $\pm$ 5 min and 144h $\pm$ 30 min after D1 infusion (D7); at 312h $\pm$ 30 min after D1 infusion (D14); before administration on D21/D28/D35 (within 0.5 h $\pm$ 5 min); before D42 infusion (within 0.5 h $\pm$ 5 min); and at 0h $\pm$ 5 min and 144h $\pm$ 30 min after D42 infusion (D48) and 312h $\pm$ 30 min after D55 infusion.

27 Follow up and record information on all adverse events from the date the subject signs the informed consent form until either the safety follow-up period or the initiation of new liver protection therapy (e.g., artificial liver support, liver transplantation), whichever occurs first.

28 Record all concomitant medications and treatments from 14 days prior to the initial administration until the safety follow-up period.

29 The AS1501-induced release of cytokines includes IL-2, IFN- γ , IL-6, TNF- α , IL-10, among others.

Visit Description	Screening Period	stage of therapy					Treatment Completed/Early Exit	Safety Follow-up	long term follow-up	
Visit Time	D-14~-1	W1	W2	W3	W4		14 days after the last administration	28 days after the last administration	W8	W12
		D1	D8	D15	D22	D28	D36	D50	D60	D90
Time Window (days)	-	-	±2	±2	±3	±3	±3	±7	±7	±7
Sign the Informed Consent Form 1	×									
Demographic Statistics Data 2	×									
Medical history, treatment history, and personal history 3	×									
Selection and Exclusion Criteria 4	×									
Height, Weight, BMI 5	×	×	×	×	×	×	×		×	×
Life Signs 6	×	×	×	×	×	×	×		×	×
Physical Examination 7	×	×	×	×	×	×	×			
Random Grouping 8		×								
Blood pregnancy test (for women of childbearing age) 9	×	×					×			
Virus Screening 10	×									
Abdominal CT (digestive system) 11	×									

Visit Description	Screening Period	stage of therapy					Treatment Completed/Early Exit	Safety Follow-up	long term follow-up	
Visit Time	D-14~-1	W1	W2	W3	W4		14 days after the last administration	28 days after the last administration	W8	W12
		D1	D8	D15	D22	D28	D36	D50	D60	D90
Time Window (days)	-	-	±2	±2	±3	±3	±3	±7	±7	±7
echocardiogram	×									
12-lead electrocardiogram (ECG)	×	×	×	×	×	×	×		×	×
Complete blood count: 13	×	×	×	×	×	×	×		×	×
Blood Biochemistry 14	×	×	×	×	×	×	×		×	×
Coagulation Function 15	×	×	×	×	×	×	×		×	×
Urinalysis 16	×	×	×	×	×	×	×		×	×
Arterial blood gas analysis 17	×	×	×	×	×	×	×		×	×
Inflammatory markers (CRP, procalcitonin): 18	×	×								
Cytokine 19		×	×	×	×					
Immunogenicity 20		×	×	×	×					
Pharmacokinetics (PK) 21		×	×	×	×					
Cytokine release induced by AS1501:30		×	×	×	×					

Visit Description	Screening Period	stage of therapy					Treatment Completed/Early Exit	Safety Follow-up	long term follow-up	
Visit Time	D-14~-1	W1	W2	W3	W4		14 days after the last administration	28 days after the last administration	W8	W12
		D1	D8	D15	D22	D28	D36	D50	D60	D90
Time Window (days)	-	-	±2	±2	±3	±3	±3	±7	±7	±7
AS1501 In vivo receptor saturation		×	×	×	×					
PD marker (TRAIL) 22	×	×	×	×	×		×			
Research on Drug Administration 23		×	×	×	×					
Child-Pugh score: 24	×	×	×	×	×	×	×		×	×
Asia-Pacific Liver Disease Research Association ACLF Research Consortium (AARC) scoring system score 25	×	×	×	×	×	×	×		×	×
MELD score for end-stage liver disease: 26	×	×	×	×	×	×	×		×	×
Diagnosis of SIRS 27	×	×	×	×	×	×	×		×	×
Event Record 28: Adverse Event	×									
Combined medications: 29	×									

- 1 Participants must sign an informed consent form prior to undergoing any study-related procedures.
- 2 Demographic data include age, gender, and ethnicity.

- 3 Medical history and personal history: This includes the disease history of previous liver disorders, as well as the medical history and treatment history of other conditions; the history of prior liver diseases should specify the exact diagnosis, diagnostic date, and previously administered therapeutic interventions. The subject's history of other diseases and treatments should be obtained and documented. Personal history encompasses smoking history, alcohol consumption history, surgical or traumatic history, allergy history, infectious disease history, and drug abuse/treatment history.
- 4 Eligibility criteria for subjects are determined during the screening phase.
- 5 Height and BMI were measured only during the screening period. Weight was measured at the following time points: screening period, before administration on D1/D8/D15/D22, D28, at the treatment completion visit, D60, and D90.
- 6 The vital sign monitoring items include: body temperature, respiration, pulse/heart rate, and blood pressure; measurements should be taken after 10 minutes of rest in a quiet environment. Measurement time points are as follows: during the screening period; within 0.5 h $\pm$ 10 min before each administration on Days D1, D8, D15, and D22; at 1 h $\pm$ 10 min, 2 h $\pm$ 10 min, and 4 h $\pm$ 20 min after infusion initiation; on Day D28; during the treatment conclusion visit; and on Days D60 and D90.
- 7 The comprehensive physical examination covers general condition, head and neck, lymph nodes, chest, abdomen, musculoskeletal system, skin, nervous system, and other areas, and will be conducted during the screening visit. During subsequent visits, a simplified examination will be performed, including but not limited to general assessment and any abnormal signs deemed significant by the investigator.
- 8 Randomization: The 72 enrolled ACLF subjects were randomly assigned in a 1:1 ratio to receive either AS1501 or placebo.
- 9 Only women of childbearing age require blood pregnancy testing; testing time points include the screening period, before administration on Day 1 (results within 3 days prior to the first dose are acceptable), and at the end-of-treatment visit. Researchers may perform additional serum pregnancy tests based on clinical indications.
- 10 Viral screening includes: hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), hepatitis C antibody (Anti-HCV), hepatitis B virus DNA (HBV-DNA), and hepatitis C virus RNA (HCV-RNA). HBV-DNA testing should be performed when HBsAg is positive or when deemed necessary by the investigator, while HCV-RNA testing should be conducted when necessary. Additionally, human immunodeficiency virus antibody (HIV) and Treponema pallidum antibody tests are included; these examinations are scheduled during the screening phase. For patients with chronic hepatitis virus infection, researchers may recommend regular follow-up monitoring of viral-related indicators.
- 11 Abdominal CT: An abdominal CT scan must be completed during the screening period. Results obtained within one month from any center with satisfactory quality are acceptable.
- 12 12-lead electrocardiogram (ECG): Includes parameters such as heart rate, RR interval, PR interval, QT interval, QRS interval, and QTc interval. The provisional testing time points are: during the screening period; within 0.5 h $\pm$ 10 min before each dose administration on days D1/D8/D15/D22; at 0 h $\pm$ 10 min, 1 h $\pm$ 10 min, 2 h $\pm$ 10 min, 8 h $\pm$ 20 min after each infusion on days D1/D8/D15/D22; at the treatment completion visit; and on days D60 and D90. If QTcF interval prolongation occurs in a subject, investigators may increase the testing frequency based on individual circumstances. Prior to each test, subjects should rest for at least 10 minutes. If blood sampling is scheduled on the same day, the ECG must be completed within 30 minutes prior to sampling. Additional ECG examinations may be performed as clinically indicated.
- 13 The complete blood count (CBC) parameters include: white blood cell count (WBC), absolute neutrophil count (ANC), neutrophil percentage, eosinophil percentage, basophil percentage, lymphocyte count (LYM), lymphocyte percentage, monocyte count (MONO), monocyte percentage, red blood cell count (RBC), hemoglobin (HGB), and platelet count (PLT). Provisional

- testing time points are: screening period; before administration on days D1/D8/D15/D22 (results within 1 day prior to administration are acceptable); D28; treatment completion visit; D60; and D90. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 14 The blood biochemical tests include: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin (ALB), pre-albumin (Pre-ALB), total bilirubin (TBIL), direct bilirubin (DBIL), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatinine (CREA), urea (UREA), uric acid (UA), potassium (K), sodium (Na), chloride (Cl), calcium (Ca), magnesium (Mg), phosphorus (P), glucose, lactate dehydrogenase (LDH), and creatine kinase. Provisional testing time points are: screening period; before dosing on days D1/D8/D15/D22 (results within 1 day prior to dosing are acceptable); D28; treatment completion visit; D60; and D90. During the treatment phase, additional tests may be performed by the investigator based on the subject's condition.
- 15 Coagulation function tests include: prothrombin time (PT), activated partial thromboplastin time (APTT), international normalized ratio (INR), fibrinogen (FIB), and prothrombin activity (PTA). Testing points are: screening period; before administration on days D1, D8, D15, and D22 (results within 1 day prior to administration are acceptable); D28; end-of-treatment visit; D60; and D90. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 16 The routine urinalysis parameters include: pH, glucose, protein, occult blood, ketones, bilirubin, urobilinogen, nitrite, white blood cells, red blood cells, and specific gravity. Testing time points are: screening period; before administration on days D1/D8/D15/D22 (results within 1 day prior to administration are acceptable); D28; treatment completion visit; D60; and D90. During the treatment phase, additional tests may be performed by the investigator based on the subject's condition.
- 17 Arterial blood gas analysis parameters include: pH, partial pressure of oxygen (PO_2), arterial oxygen saturation (SaO_2), partial pressure of carbon dioxide (PCO_2), and lactate levels. Measurement time points are: screening period; before administration on days D1/D8/D15/D22 (results within 1 day prior to administration are acceptable); D28; end-of-treatment visit; D60; and D90. During the treatment phase, additional tests may be performed by the investigator based on the subject's clinical condition.
- 18 Inflammatory markers: including C-reactive protein (CRP) and procalcitonin; Provisional testing time points: screening period, before administration on Day 1 (if the test is performed within ≤ 3 days of the screening period, it may be omitted). During the treatment period, additional tests may be conducted by the investigator based on the subject's condition.
- 19 Cytokines: At least TNF- α and IL-6 must be included; investigators may add additional cytokine assays based on the available testing panels at each center, taking into account the subject's condition. Sampling time points: samples shall be collected before each infusion (within $0.5\text{ h} \pm 5\text{ min}$) on days D1/D8/D15/D22, at $0\text{ h} (\pm 30\text{ min})$ after each infusion, and at $24\text{ h} (\pm 30\text{ min})$ post-infusion. During the treatment period, additional tests may be performed by investigators based on the subject's clinical status.
- 20 Immunoorigidity testing: Before administration of D1/D8/D15/D22 (within $0.5\text{ h} \pm 5\text{ min}$). During the trial, subsequent immune origin sampling points may be adjusted based on preliminary immunoorigidity test results.
- 21 Pharmacokinetic sample collection: Subjects were administered PK blood samples at trough concentrations before infusion on days D8, D15, and D22 (within $0.5\text{ h} \pm 5\text{ min}$), and PK blood samples at peak concentrations immediately after infusion completion on days D1 and D22 (at $0\text{ h} \pm 5\text{ min}$). A total of five PK sampling time points were designated.
- 22 TRAIL blood samples: Primarily used to measure serum TRAIL levels and membrane-bound TRAIL levels on NK cells. Blood samples for TRAIL measurement must be collected at six designated time points: during the screening phase, before drug administration on days D1/D8/D15/D22 (within $0.5\text{ h} \pm 5\text{ min}$), and at the end of the treatment visit.

- 23 Study Drug Administration: The 72 enrolled ACLF subjects received the study drug at appropriate doses and frequencies according to a randomization ratio of 1:1 (AS1501:placebo). Specific dosing regimens and administration frequencies will be determined based on the results from Phase IIa trials.
- 24 Child-Pugh score: Liver function should be graded using the modified Child-Pugh classification system. Reference parameters include hepatic encephalopathy, ascites, total bilirubin, albumin, and prolonged prothrombin time. Based on these parameters, the scores are classified as: Grade A (5–6 points), Grade B (7–9 points), or Grade C (≥ 10 points). Measurement time points include: screening period; before administration on days D1/D8/D15/D22; day D28; at treatment completion visit; day D60; and day D90.
- 25 ACLF Research Alliance (AARC) scoring system developed by the Asia-Pacific Liver Disease Research Association. Time points: screening period; before dosing on D1/D8/D15/D22; D28; treatment completion visit; D60; D90.
- 26 MELD score for end-stage liver disease. Time points: screening period, before dosing on days D1/D8/D15/D22, day D28, treatment completion visit, day D60, and day D90.
- 27 SRIS: Includes body temperature, heart rate, respiratory rate, and white blood cell count. Time points: screening period; before administration on D1/D8/D15/D22; D28; treatment completion visit; D60; D90.
- 28 Follow up and record information on all adverse events from the date the subject signs the informed consent form until either the safety follow-up period or the initiation of new liver protection therapy (e.g., artificial liver support, liver transplantation), whichever occurs first.
- 29 Record all concomitant medications and treatments administered from 14 days prior to the initial dose administration until the end of treatment.
- 30 The AS1501-induced release of cytokines includes IL-2, IFN- γ , IL-6, TNF- α , IL-10, among others.

2. Introduction

2.1. Theoretical Basis for the Study

2.1.1. Slow-onset acute liver failure

Acute-on-chronic liver failure (ACLF) is a syndrome that develops on the basis of chronic liver disease, triggered by various precipitating factors, characterized by acute worsening of jaundice and coagulation disorders as manifestations of liver failure. It may be complicated by complications such as hepatic encephalopathy, ascites, electrolyte disturbances, infections, hepatorenal syndrome, hepatopulmonary syndrome, and extrahepatic organ failure. According to the 2018 edition of China's "Guidelines for Diagnosis and Treatment of Liver Failure" [1], its diagnostic criteria include: rapid progression of jaundice with serum TBil ≥ 10 ULN or a daily increase ≥ 17.1 $\mu\text{mol/L}$; presence of bleeding manifestations with PTA $\leq 40\%$ (or INR ≥ 1.5). Based on the underlying chronic liver disease, ACLF is classified into three types: Type A: acute-on-chronic liver failure occurring on the basis of chronic non-cirrhotic liver disease; Type B: acute-on-chronic liver failure occurring on the basis of compensated cirrhosis, typically within 4 weeks; Type C: acute-on-chronic liver failure occurring on the basis of decompensated cirrhosis.

ACLF is a significant global medical challenge [2]. In a large retrospective study, the prevalence of ACLF in patients with decompensated cirrhosis was 35% (95% CI: 33%–38%), with the highest prevalence observed in South Asia at 65% (95% CI: 47%–84%) and 15% in East Asia (95% CI: 13%–18%) [3]; its true prevalence may be underestimated. In China, the prevalence of ACLF among patients with decompensated cirrhosis due to hepatitis B infection is approximately 34%. With active prevention and effective control of chronic hepatitis B virus infection, the incidence of ACLF in China has declined annually. A prospective cross-sectional study covering all hospitalized patients (2005–2014) reported an overall incidence rate of ACLF as 2.53 per 100,000 person-years (95% CI: 2.16–2.91), a significant decrease from the 2005 rate of 3.35 per 100,000 person-years. The incidence was notably higher in males than in females (3.83 vs. 1.25 per 100,000 person-years). The peak incidence in males occurred around ages 35–39 and again between 65 and 69 years (a smaller peak), while females exhibited two peaks: ages 50–54 and 65–69 [4].

In China, the main causes of ACLF include hepatitis B virus hepatitis, alcohol, and hepatotoxic substances such as drugs (including traditional Chinese medicine, anti-tuberculosis drugs, antibiotics, antineoplastic agents, and nonsteroidal anti-inflammatory drugs). A prospective cross-sectional study in China involving all hospitalized patients (2005–2014) revealed that the various underlying chronic liver diseases among ACLF patients were: hepatitis B virus hepatitis, autoimmune liver disease, alcoholic liver disease, and drug-induced liver injury, accounting for 91%, 3%, 2.74%, and 1.19% respectively [4].

Acute coronary limb failure (ACLF) is characterized by a rapid clinical progression, diverse clinical manifestations, and high short-term mortality [2]. The 28-day mortality rate ranges from approximately 30% to 45% (95% CI: 41%–48%), while the 90-day mortality rate is about 58% (95% CI: 55%–61%) [3]. Moreover, the higher the number of concurrent organ failures and the more severe the disease severity, the greater the mortality risk. Over the past two decades, no significant reduction in mortality rates among ACLF patients in Asian populations has been observed; the mortality rate remains close to 50% [5].

The pathogenesis of ACLF is comprehensive and complex, and has not yet been fully elucidated. It is typically explained through the PIRO concept, which encompasses susceptibility, injury stressors, host response, and organ failure [6]. ACLF develops when a patient with underlying liver disease experiences chronic liver injury and subsequently suffers from various acute intrahepatic or extrahepatic injuries, leading to acute hepatic decompensation manifested as hyperbilirubinemia, coagulation disorders, hepatic encephalopathy, and ascites. The systemic response induced by such injury can be divided into two components: First, following acute liver injury, dysregulation of cellular immunity and the hepatic reticuloendothelial system leads to diminished capacity of the liver to synthesize various anti-infective proteins, thereby increasing the risk of bacterial infections—such as spontaneous bacterial peritonitis caused by gut microbiota translocation—as well as infections in other systems (e.g., urinary tract infections, pneumonia, and skin/soft tissue infections)—resulting in elevated levels of PAMPs (pathogen-associated molecular patterns) in the patient's circulation. Second, necrotic hepatocytes release substantial amounts of DAMPs (damaged-associated molecular patterns) into the systemic circulation, where these PAMPs bind to pattern recognition receptors (PRRs) specific to innate immune cells, activating the immune system and triggering the release of numerous cytokines—including TNF,

IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, and IFN- γ — which induce a "cytokine storm" that exacerbates systemic inflammatory responses. Furthermore, DAMPs released from injured hepatocytes intensify this systemic response, leading to hyperinflammation and subsequent damage to extrahepatic organs. Following an initial phase of excessive systemic inflammation, ACLF progresses to an immunodepleted or immunoparalytic state in its advanced stages, further aggravating bacterial infections and ultimately culminating in multi-organ failure and patient mortality [7][8][9][10][11][12][13][14][15].

Currently, there is no specific targeted treatment for ACLF patients. The main therapeutic approaches include comprehensive medical therapy, artificial liver support systems, liver transplantation, and stem cell transplantation. Comprehensive medical therapy serves as a fundamental basis for managing liver failure, yet there is a lack of specific drugs or methods tailored for this condition. Artificial liver, also known as an artificial liver support system, is primarily categorized into non-biological, biological, and hybrid types. Its primary functions include eliminating various harmful substances from the body, replenishing essential nutrients, temporarily replacing partial liver functions, improving the internal environment, and creating conditions for hepatocyte regeneration and functional recovery, or awaiting the opportunity for liver transplantation [16]. However, non-biological artificial livers mainly focus on removing toxic substances from the patient's blood; they cannot fully perform hepatic metabolic, secretory, or synthetic functions, nor can they fundamentally reverse liver pathology, thus failing to significantly improve survival rates in patients with advanced liver failure [17]. Although biological and hybrid artificial livers possess liver-specific detoxification, biosynthesis, and transformation capabilities with significant application potential, their clinical use is largely constrained by factors such as cell sources, cell culture techniques, and bioreactors [18]. Liver transplantation remains the most effective treatment for mid-to-late-stage liver failure when comprehensive medical therapy and artificial liver support systems prove ineffective. However, it faces two major challenges: donor shortages, particularly severe in China, and the need for long-term immunosuppressive therapy in recipients, which is costly and associated with numerous side effects [19]. With advancements in modern medicine and ongoing progress in stem cell research, although stem cell therapy for liver failure has demonstrated certain therapeutic efficacy, numerous challenges remain unresolved: (1) optimal timing for transplantation; (2) appropriate number of transplanted cells; (3) optimal transplantation routes; (4) the impact of the patient's immune status, endotoxins, and elevated bilirubin levels on

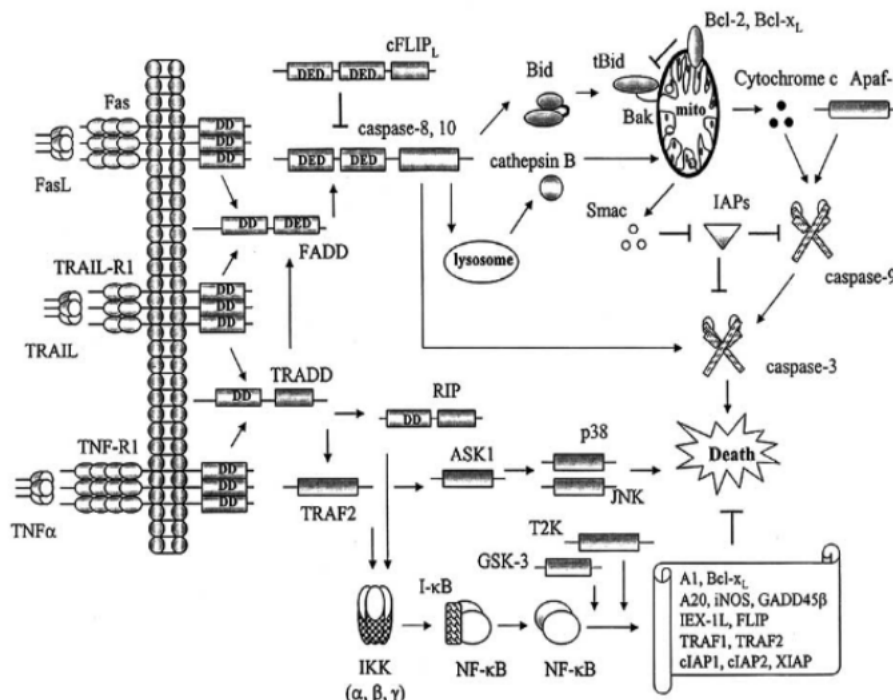


Figure 1 Mechanisms of death receptor-mediated endogenous and exogenous apoptosis in hepatocytes [24]

TRAIL, also known as Apo2L, was first identified by Wiley et al. in 1995 and represents the third member of the TNF family of apoptosis-inducing molecules following TNF and FasL. As a type II membrane protein, TRAIL comprises a non-conserved N-terminal (cytoplasmic) domain and a highly conserved C-terminal (extracellular) domain, which bind to DR4 and DR5 to activate their cytoplasmic death domains, form a homotrimer, and recruit the fas-related death domain (FADD) into the death-inducing signaling complex (DISC) [25][26][27]. The DISC interacts with caspase-8 and caspase-10, triggering an apoptotic signaling cascade via receptor-dependent or mitochondria-dependent pathways. In the mitochondria-dependent pathway, active caspases-8/10 convert the inhibitory Bcl-2 protein with a BH3 domain (Bid) into tBid [28][29]. The synergistic interaction between BCL2-associated X (BAX) and Bcl-2 homologous antagonist/killer (BAK) induces mitochondrial translocation of tBid and cytoplasmic release of cytochrome c, subsequently leading to the assembly of procysteine-9 and apoptosis protease activator factor-1 (APAF-1) to form an apoptotic complex that ultimately activates caspases-3/6/7. In the receptor-dependent pathway, activated caspase-8 directly induces apoptosis via the executioner cysteine protease [30][31]. The activated caspase cysteine-aspartate triggers apoptosis-related events, including DNA fragmentation and the lysis of cytoplasmic, cytoskeletal, and nuclear proteins [32]. See the figure below for details.

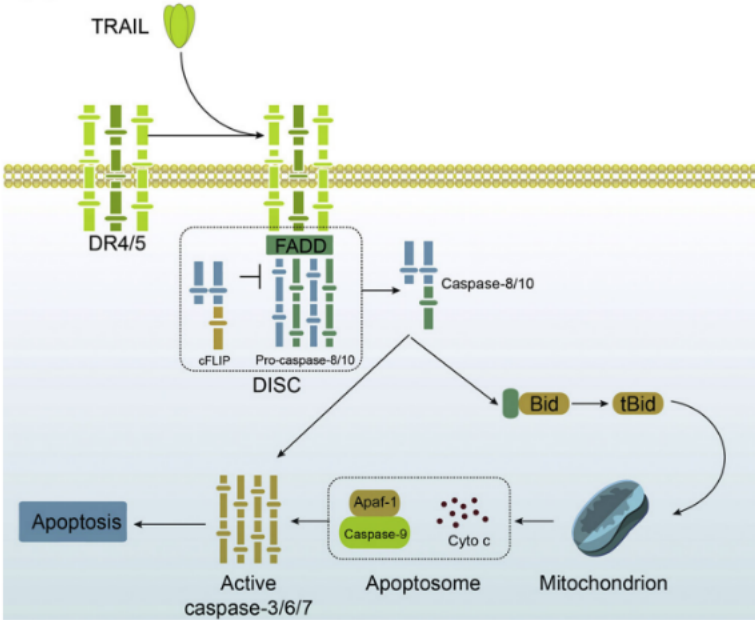


Figure 12 Classic signaling pathways of TRAIL-mediated endogenous and exogenous apoptosis in hepatocytes

Studies on liver injury have demonstrated that TRAIL plays a pivotal role in diseases such as viral hepatitis, fatty liver disease, and autoimmune liver disorders [32]. The mechanisms of TRAIL involvement in liver diseases include: 1) Inhibition of HBV replication and expression by downregulating liver-enriched transcription factors HNF4 α , PPAR α , and RXR α . Similar to HBV, TRAIL levels are elevated in patients with HCV infection compared to healthy controls, and its upregulation is associated with disease progression; 2) Soluble TRAIL levels are higher in patients with nonalcoholic fatty liver disease (NAFLD) than in healthy controls, and these levels exhibit a positive correlation with triglyceride levels [33]. TRAIL deficiency induces systemic insulin resistance, enhances hepatic cholesterol and glucose synthesis, predisposes the liver to triglyceride accumulation and liver injury, thereby exacerbating NAFLD severity [33][34]. TRAIL significantly mitigates diet-induced metabolic abnormalities and counteracts impaired glucose tolerance and NAFLD [35]; 3) It suppresses autoimmune inflammation, maintains immune homeostasis, and modulates immune responses (closely associated with CD95L or CD30L [36]). In a study on primary biliary cholangitis (PBC), TRAIL does not induce apoptosis of inflammatory cells but suppresses autoimmune inflammation by inhibiting DNA synthesis and blocking the lymphocyte cell cycle progression; 4) TRAIL plays a critical role in ameliorating hepatic fibrosis and cirrhosis by selectively inducing apoptosis of activated hepatic stellate cells [37][38].

Both in vitro and in vivo studies demonstrate that normal hepatocytes are tolerant to TRAIL-mediated apoptosis, whereas chronic viral infections, steatosis, and exposure to toxic substances can upregulate TRAIL expression in hepatocytes [22]. Furthermore, research on the immune mechanisms underlying liver failure indicates that the activation of immune cells—including Kupffer cells, dendritic cells, natural killer cells, cytotoxic T lymphocytes, and regulatory T cells—as well as their cytokine production, plays a significant role in the pathogenesis of liver failure. During liver injury, TRAIL-expressing natural killer cells exhibit potent hepatotoxic effects [39].

2.2. background

2.2.1. research on drug

2.2.1.1. mechanism of action

The investigational drug AS1501 is a Class 1 therapeutic biologic that competitively binds to TRAIL on the cell surface via its receptor TRAIL-R2 (DR5). The SDR5 domain of AS1501 can bind to its ligand TRAIL; however, due to the absence of a transmembrane region and an intracellular death domain, SDR5 cannot transmit apoptotic signals into the cell. Consequently, SDR5 competes with death receptors on the cell membrane for TRAIL binding, thereby inhibiting TRAIL-mediated apoptosis. See the figure below for details.

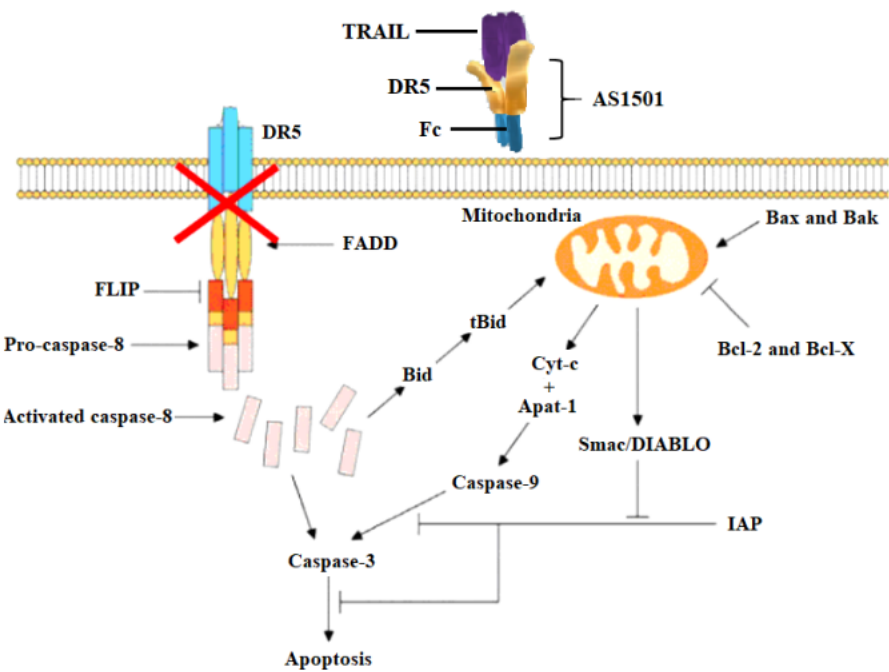


Figure 23: AS1501 binds to TRAIL and blocks apoptosis signaling

Liver damage caused by viral hepatitis and immune-mediated hepatitis is primarily mediated by cellular immunity, where immune cells release cytokines such as TRAIL, FasL (CD95L), TNF- α , and interferon- γ (IFN- γ), which induce apoptosis through various signaling pathways. Extensive experimental evidence demonstrates that although multiple signaling pathways can trigger apoptosis, their downstream mechanisms are shared, involving the activation of caspases and DNA degradation. Furthermore, during hepatocyte death induction, TRAIL and other signaling pathways exhibit mutual dependence and synergy; effective hepatocyte killing by immune cells requires the simultaneous presence of both TRAIL and these other pathways. In other words, blockade of TRAIL prevents other cytokines from activating sufficient downstream (shared) caspases, thereby inhibiting DNA degradation and hepatocyte death. For instance, in a human-type hepatitis B virus transgenic mouse model, blocking only TRAIL without affecting other cytokines effectively prevents hepatocyte DNA degradation and death. Similarly, in ConA-induced acute immune hepatitis, inhibition of TRAIL or knockout of the TRAIL gene significantly reduces hepatocyte DNA degradation and death.

In conclusion, hepatocyte death induced by viral or immune hepatitis is mediated by TRAIL and several interdependent cytokines; blocking TRAIL alone can effectively prevent hepatocyte death caused by viral or immune hepatitis.

2.2.1.2. physicochemical property

The detailed specifications of the AS1501 are shown in the table below.

Chinese Common Name	AS1501 for Injection
English Generic Name	AS1501 for Injection
active ingredient	Human recombinant AS1501 fusion protein
Excipients	Sodium dihydrogen phosphate monohydrate, Disodium hydrogen phosphate dihydrate, Sodium chloride, Mannitol, Polysorbate 20
Structure Formula	The dimeric fusion protein consists of the extracellular domain fragment of truncated human death receptor 5 (DeathReceptor5) combined with the Fc segment (including the hinge, CH2, and CH3 regions) of human IgG1.
formula weight	Approximately 79 KD
physical characteristics	The drug has a pH of 6.7 – 7.3; an osmotic pressure molar concentration of 270 – 330 mOsmol/kg; and moisture content not exceeding 3.0%.
stability	The validity period is tentatively set at 3 years
the form of a drug	Lyophilized Powder for Injection

shape and properties	White loose bodies
specifications	25 mg per vial
storage procedures	Seal tightly, store away from light, refrigerated at 2°C–8°C

2.2.2. Overview of Preclinical Studies

2.2.2.1. Pharmacodynamic Studies

2.2.2.1.1. In vitro pharmacodynamic studies

In vitro studies have demonstrated through the following experiments that AS1501 inhibits the cytotoxic activity of TRAIL and specifically binds to TRAIL.

1) AS1501 in vitro assay for evaluating biological activity inhibition of TRAIL cytotoxicity

The AS1501 in vitro bioactivity assay for evaluating its inhibition of TRAIL-mediated cytotoxicity was conducted using multiple cell lines to comprehensively assess AS1501's biological activity across different tissue cells. After incubation with TRAIL protein, the viable cell count was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) assay, with TRAIL cytotoxicity results evaluated based on the A490/A630 absorbance ratio.

The experimental results demonstrate that TRAIL exhibits potent apoptotic inducing effects on human hepatocellular carcinoma cells (HepG2), human leukemia T lymphocytes (Jurkat), and mouse fibroblasts (L929), with high sensitivity, as evidenced by EC50 values all falling within the ng range.

2) Detection experiment for the specificity of AS1501 binding to TRAIL

In the experiment, the MTT assay was used to determine whether AS1501 could inhibit TNF-α-induced apoptosis, thereby assessing its potential cross-reactivity with TNF-α; the ELISA assay was employed to evaluate whether AS1501 could bind to Fas L, thereby determining its potential cross-binding activity against Fas L.

The experimental results demonstrate that at physiological concentrations, AS1501 exhibits no cross-reactivity with TNF-α or Fas L, highlighting the specificity of AS1501 in binding to TRAIL.

3) Detection experiment of AS1501 blocking TRAIL-induced apoptosis

In the experimental study evaluating AS1501's inhibition of TRAIL-induced apoptosis, HepG2 cells were used as test cells to assess TRAIL-mediated apoptosis in these cells. The experimental groups included high-concentration AS1501 group (35 ng/mL), medium-concentration group (3.5 ng/mL), low-

concentration group (0.35 ng/mL), and a blank control group (without AS1501). Changes in caspase cascade proteins were detected using Western blotting.

The experimental results demonstrate that AS1501 can inhibit the binding of TRAIL to death receptors on the cell membrane, thereby downregulating the activation of intracellular caspase cascade proteins and reducing apoptosis.

4) Affinity experiments of TRAIL proteins from different species with AS1501

In affinity assays comparing the TRAIL protein with AS1501 across different species, AS1501 was tested for binding affinity with human TRAIL, macaque TRAIL, and mouse TRAIL; the results are presented in the table below.

Table 1 Affinity measurements and kinetic data of TRAIL versus AS1501 across different species

part	analyte	ka (1/Ms)	kd (1/s)	KD (M)	Chi² (RU²)
AS1501	human being TRAIL	2.91E+06	3.01E-04	1.03E-10	2.24E-01
	monkey TRAIL	2.89E+06	2.63E-04	9.12E-11	1.88E-01
	mouse TRAIL	1.73E+04	3.83E-04	2.22E-08	7.02E-01

The affinity data demonstrate that AS1501 exhibits strong binding activity with TRAIL; both human and monkey TRAIL show similar binding affinities to AS1501, which are higher than those observed between mouse TRAIL and AS1501. This indicates that the pharmacodynamic and safety data obtained from mouse and monkey models can serve as references for human studies, with results from the monkey model being particularly closer to those in humans.

➤ Affinity experiments between TRAIL protein and TRAIL-R2

In the affinity assay for TRAIL protein and TRAIL R2, the binding affinity of human TRAIL to TRAIL R2 (Fc chimera) was 4.23×10^{-11} M.

➤ Affinity determination of Fcγ receptor protein with AS1501

In the affinity assay between Fcγ receptor protein and AS1501, Fcγ RI CD64 exhibited excellent binding affinity with AS1501.

5) TRAIL biomarker as a companion diagnostic for AS1501

The study on TRAIL biomarkers as adjunct diagnostic tools for AS1501 demonstrated that in patients with liver failure, TRAIL levels exhibited positive correlations with hepatic function

parameters—alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—and negative correlations with prothrombin time activity (PTA), with these relationships being particularly pronounced during dynamic monitoring. This highlights the remarkable consistency between TRAIL levels and the dynamic changes of aforementioned liver injury and severity indicators, underscoring the significant predictive value of TRAIL monitoring for both hepatic inflammatory damage and disease severity.

2.2.2.1.2. In vivo Pharmacodynamic Studies

To demonstrate the efficacy of AS1501 in treating liver injury and/or liver failure caused by viral, drug-induced, or autoimmune etiologies, we reviewed the literature and conducted a series of formal clinical trials for comprehensive analysis.

1) **Pharmacodynamic Evaluation of AS1501 in an Acetylamino-phenol-Induced Mouse Liver Failure Model**

In this experiment, the pharmacodynamics of AS1501 was evaluated in a phenylacetamide-induced mouse liver failure model. During the study, no deaths or near-death cases were observed in the control group. The survival rate of mice with APAP-induced models treated with normal saline decreased to 16.7%; however, 15.0 mg/kg of AS1501 significantly improved the survival rate to 55.6%. Moreover, within the dose range of 5.0–15.0 mg/kg, the therapeutic efficacy of AS1501 showed a positive correlation with the drug dosage. The positive drug NAC demonstrated favorable therapeutic effects at 250 mg/kg, whereas the anti-TNF-α monoclonal antibody at 10.0 mg/kg exhibited no significant therapeutic effect. Statistical tables and survival curve plots of AS1501's efficacy in the phenylacetamide-induced mouse liver failure model are presented in Table 2 and Figure 4, respectively.

Under these experimental conditions, the test substance AS1501 was capable of treating APAP-induced acute liver failure in mice and improving their survival rate.

Table 1 Statistical analysis of survival rates of mice in the AS1501 treatment group in a paracetamol-induced mouse liver failure model

group		Number of animals (only)	Number of survivors after 28 days of administration (only)	Survival Rate	P price (Compared to G3)
G1	Phosphate Buffered Saline (PBS)	20	20	100%	< 0.0001

G2	Phosphate saline-AS1501 (15 mg/kg)	20	20	100%	< 0.0001
G3	APAP – Normal saline	30	5	16.7%	/
G4	APAP-NAC (250 mg/kg)	28	24	85.7%	< 0.0001
G5	APAP-Anti-TNF-α (10mg/kg)	21	7	33.3%	0.1676
G6	APAP-AS1501 (5 mg/kg)	26	8	30.8%	0.4035
G7	APAP-AS1501 (10 mg/kg)	26	11	40.7%	0.0466
G8	APAP-AS1501 (15 mg/kg)	27	15	55.6%	0.0025

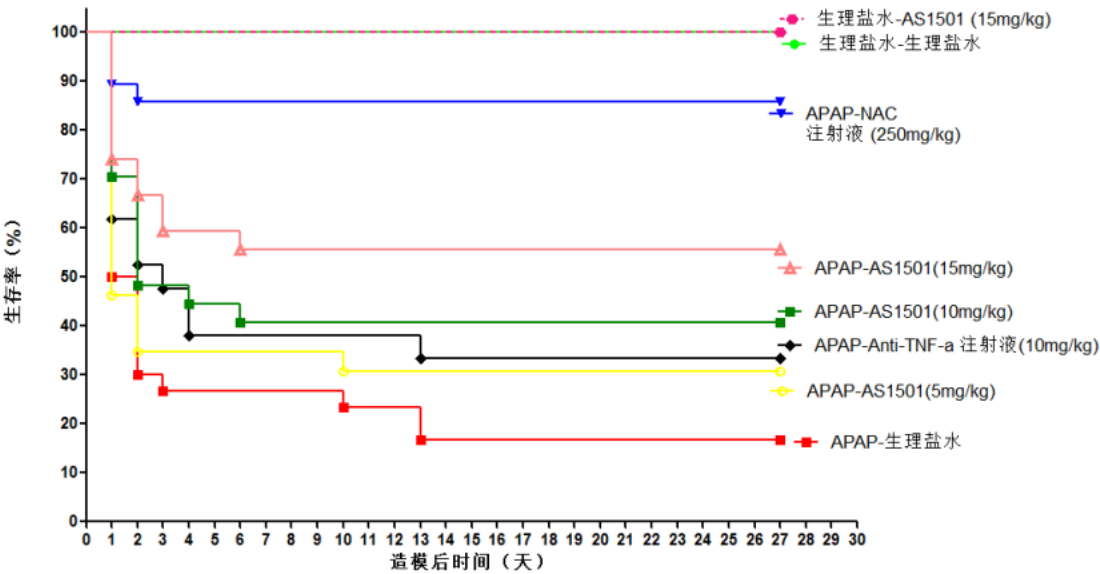


Figure 3 Survival curve of AS1501 in a paracetamol-induced mouse liver failure model

2) Pharmacodynamic Evaluation of AS1501 in a Paracetamol-Induced Drug-Induced Liver Injury Model in Mice

In this study, the pharmacodynamics of AS150 were evaluated in a mouse model of drug-induced liver injury induced by acetaminophen. During the trial, no deaths or near-death conditions related to the test substance were observed in any animals.

General observations revealed: (1) During animal dissection, no pathological changes were observed in the livers or spleens of 1–2 groups of animals, indicating that the dose of AS1501 at 27.0 mg/kg is safe for mice. (2) The negative control group in the modeling cohort exhibited severe hepatic

lesions, confirming successful establishment of drug-induced liver injury models. (3) Liver lesions in the test dose groups were less severe than those in the negative control group, demonstrating therapeutic efficacy of AS1501 at a dose of 9.0 mg/kg against APAP-induced hepatitis in mice.

Biochemical findings revealed: (1) ALT and AST levels in the non-modeling groups G1 and G2 remained within normal ranges, indicating that AS1501 at a dose of 27.0 mg/kg is safe for mice. (2) ALT and AST levels in mice in the modeling group administered with physiological saline were significantly higher than those in the non-modeling group, confirming successful model establishment. Significant reductions in ALT and AST levels were observed in all dosage groups of the test substance, demonstrating that AS1501 exhibits therapeutic efficacy against APAP-induced drug-induced liver injury at a dose of 9.0 mg/kg.

Pathological section H&E staining results showed: (1) No pathological areas were observed in the non-modeling groups G1 and G2, indicating that doses of AS1501 up to 27.0 mg/kg are safe. (2) Compared with the non-modeling groups G1 and G2, the modeling group exhibited significant hepatic injury areas, with the negative control group G3 showing the largest affected area; the injury areas in the test substance group were markedly smaller than those in G3, demonstrating that AS1501 exhibits therapeutic efficacy against APAP-induced acute liver injury in mice at doses of 3.0 mg/kg, 9.0 mg/kg, and 27.0 mg/kg. The ALT and AST values and level plots for each group are presented in Table 3 and Figure 5.

Table 1 ALT and AST levels of AS1501 in a paracetamol-induced drug-induced liver injury model in mice

group		ALT (U/L)	P price (Compared to Group G3)	AST (U/L)	P price (Compared to Group G3)
G1	Phosphate Buffered Saline (PBS)	56.76±8.608	< 0.0001	161.2±43.84	< 0.0001
G2	Phosphate Buffered Saline-AS1501 (27 mg/kg)	51.90±4.118	< 0.0001	111.9±7.509	< 0.0001
G3	APAP – Normal saline	10619±874.2	/	5151±535.9	/
G4	APAP –NAC (27 mg/kg)	8331±420.3	0.0159	4167±273.4	0.0906
G5	APAP-AS1501 (3 mg/kg)	8775±708.4	0.1061	4241±462.1	0.2033

G6	APAP-AS1501 (9 mg/kg)	7654±596.4	0.0092	3727±368.3	0.0384
G7	APAP-AS1501 (27 mg/kg)	10926±954.1	0.8130	6061±865.8	0.3722

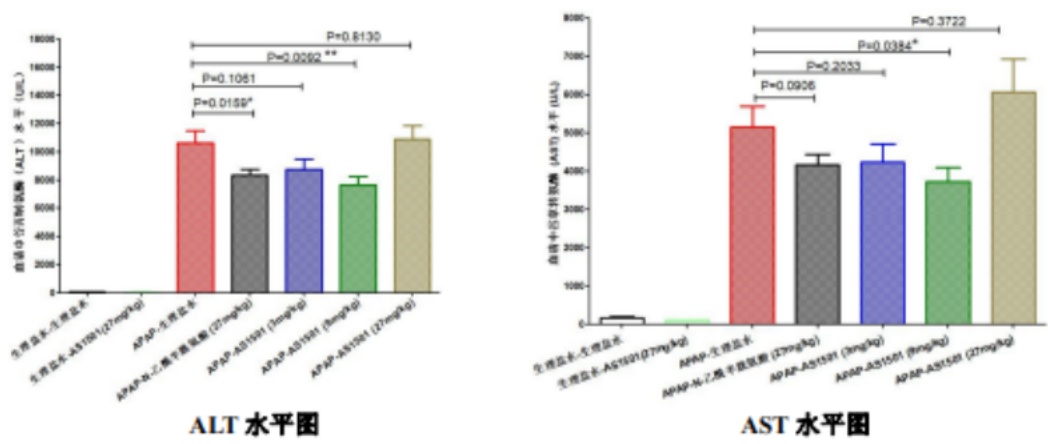


Figure 3: Comparison of ALT and AST levels

1) Pharmacodynamic evaluation of AS1501 in a mouse liver failure model induced by jack bean protein A (ConA)

In the pharmacodynamic evaluation study of AS1501 in a mouse liver failure model induced by jack bean protein A, no deaths or near-death cases were observed in the non-modeling groups (Groups 1–2). Under non-modeling conditions, mice did not die at the test dose of 30.0 mg/kg, indicating that this dose is safe for mice. In the Con A-induced model with saline administration, the survival rate of mice decreased to 2.9%. Under modeling conditions, the test group (AS1501 doses of 10.0 mg/kg, 20.0 mg/kg, and 30.0 mg/kg) showed a statistically significant improvement in survival rates compared to the negative control group, with effects superior to those of the positive control group. Specifically, 30.0 mg/kg of AS1501 increased survival rates to 32.3%, while the positive drug ganlixin at 15 mg/kg also demonstrated therapeutic efficacy, albeit less pronounced than 10.0 mg/kg of AS1501. Test doses ranging from 10.0 to 30.0 mg/kg exhibited significant therapeutic effects in the Con A-induced acute liver failure model in mice. Details are presented in Table 4 and Figure 6.

Therefore, under these experimental conditions, the test substance AS1501 can treat Con A-induced acute liver failure in mice and improve their survival rate.

Table 1: Statistics on mouse survival rates in the jack bean protein A-induced mouse liver failure model

group		Number of animals (only)	After 33 days of administration Number of Survivors (only)	Survival Rate	P price (Compared to G3)
G1	Phosphate Buffered Saline (PBS)	20	20	100%	< 0.0001
G2	Phosphate saline-AS1501 (30.0 mg/kg)	20	20	100%	< 0.0001
G3	With A-fysiologic saline	34	1	2.9%	/
G4	Con A-AS1501 (10.0 mg/kg)	33	10	30.3%	0.0026
G5	Con A-AS1501 (20.0 mg/kg)	31	9	29.0%	0.0341
G6	Con A-AS1501 (30.0 mg/kg)	31	10	32.3%	0.0069
G7	With A-ganlixin (15.0 mg/kg)	33	8	24.2%	0.0100

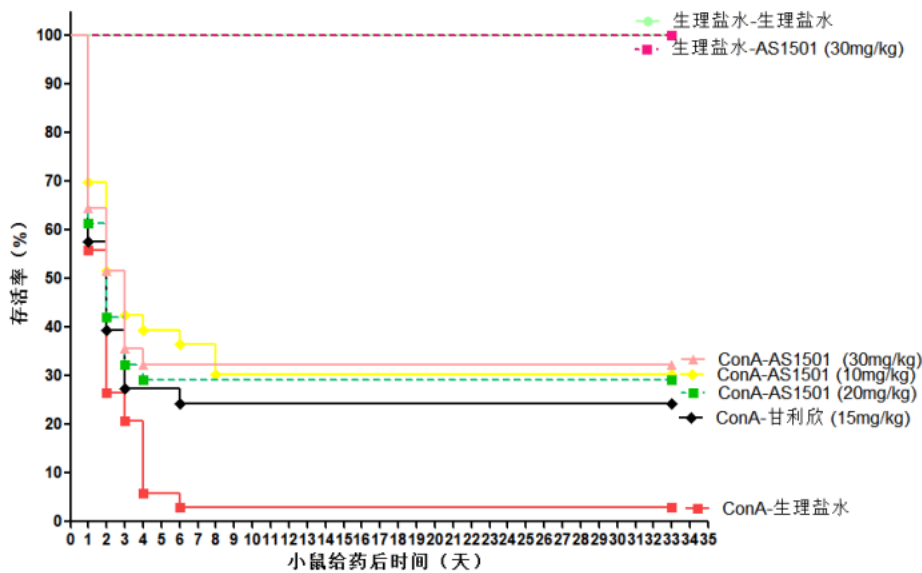


Figure 3 Survival curve of AS1501 in a mouse liver failure model induced by jack bean protein A

1) Pharmacodynamic evaluation of AS1501 in a mouse autoimmune hepatitis model induced by jack bean protein A

In the pharmacodynamic evaluation study of AS1501 in a mouse autoimmune hepatitis model induced by jack bean protein A, the following observations were made: (1) During the trial period, although all animals exhibited weight loss, no abnormal changes were observed compared to the non-modeling groups G1 and G2. (2) Upon anatomical examination, no pathological lesions were detected

in the livers or spleens of animals in groups G1–G2, indicating that the dose of AS1501 at 27.0 mg/kg was safe for mice. (3) Severe pathological lesions were observed in the livers and spleens of animals in the modeling group G3, confirming the success of the Con A-induced hepatitis model. (4) The pathological lesions in the livers and spleens of animals in the test group were less severe than those in the negative control group G3, demonstrating that the doses of AS1501 at 3.0 mg/kg, 9.0 mg/kg, and 27.0 mg/kg exhibited therapeutic effects against liver injury in mice.

The biochemical parameters alanine aminotransferase (ALT) and aspartate aminotransferase (AST) results showed: (1) In the non-modeling groups G1 and G2, ALT and AST levels remained within the normal range, indicating that the dose of AS1501 at 27.0 mg/kg was safe for mice. (2) Elevated ALT and AST levels in mice treated with physiological saline during modeling confirmed successful model establishment. The ALT and AST levels in the high-and medium-dose groups G7 and G6 were significantly lower than those in the negative control group G3, demonstrating statistical significance, which demonstrates that AS1501 exhibits therapeutic efficacy against Con A-induced autoimmune hepatitis at doses of 9.0 mg/kg and 27.0 mg/kg. Moreover, the most pronounced reduction in ALT and AST levels was observed at the 27.0 mg/kg dose, suggesting that this dosage yields optimal therapeutic effects under experimental conditions.

Pathological section H&E staining results showed: (1) No pathological areas were observed in the non-modeling groups G1 and G2, indicating that AS1501 at a dose of 27.0 mg/kg is safe for mice. (2) Compared with the non-modeling groups G1 and G2, significant liver injury areas were evident in the modeling groups G3–G7, with the largest injury area observed in the negative control group G3; the injury areas in the test groups G5 –G7 were significantly smaller than those in G3, demonstrating therapeutic efficacy of AS1501 at doses of 3.0 mg/kg, 9.0 mg/kg, and 27.0 mg/kg against Con A-induced acute liver injury in mice. ALT and AST data are detailed in Table 5 and Figure 7.

The aforementioned experimental results demonstrate that under these conditions, AS1501 exhibits therapeutic efficacy in a Con A-induced murine autoimmune hepatitis model, with its therapeutic effect showing a positive correlation with dosage within the range of 3.0–27.0 mg/kg.

Table 1 ALT and AST values of AS1501 in a mouse autoimmune hepatitis model induced by jack bean protein A

group	ALT (U/L)	P-value (compared to Group G3)	AST (U/L)	P-value (compared to
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					Group G3)
G1	Phosphate Buffered Saline (PBS)	60.24±3.856	< 0.0001	125.60±12.46	< 0.0001
G2	Phosphate Buffered Saline-AS1501 (27 mg/kg)	66.16±5.512	< 0.0001	139.70±13.69	< 0.0001
G3	With A-fysiologic saline	3047.00±395.5	/	2299.00±335.4	/
G4	With A-glycyrrhizic acid (27 mg/kg)	1475.00±559.2	0.0277	1142.00±530.3	0.0733
G5	Con A-AS1501 (3 mg/kg)	2227.00±467.2	0.1873	1551.00±350.0	0.1316
G6	Con A-AS1501 (9 mg/kg)	1388.00±333.7	0.0026	925.4±208.2	0.0010
G7	Con A-AS1501 (27 mg/kg)	1448.00±257.0	0.0015	976.9±166.0	0.0010

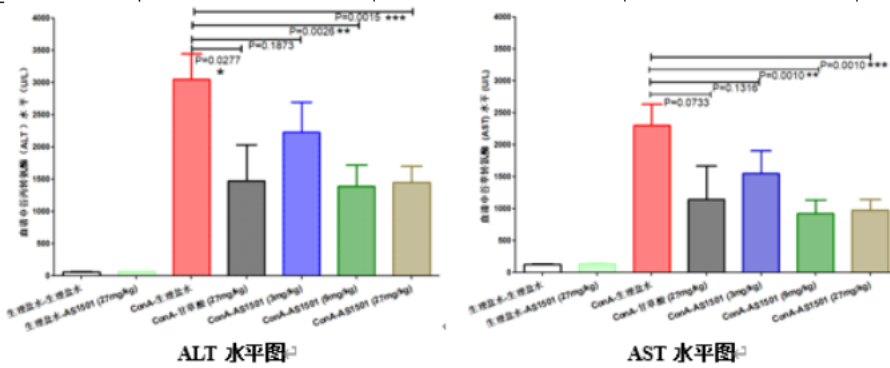


Figure 3: Comparison of ALT and AST levels

2) Research on the Immunomodulatory Molecular Mechanisms of AS1501 in Drug-Induced Hepatitis and Acute Liver Failure

The aforementioned animal studies demonstrate that AS1501 is effective against drug-induced hepatitis and acute liver failure. To further elucidate the mechanism of action of AS1501, we conducted additional in vivo studies to investigate its immunomodulatory molecular mechanisms.

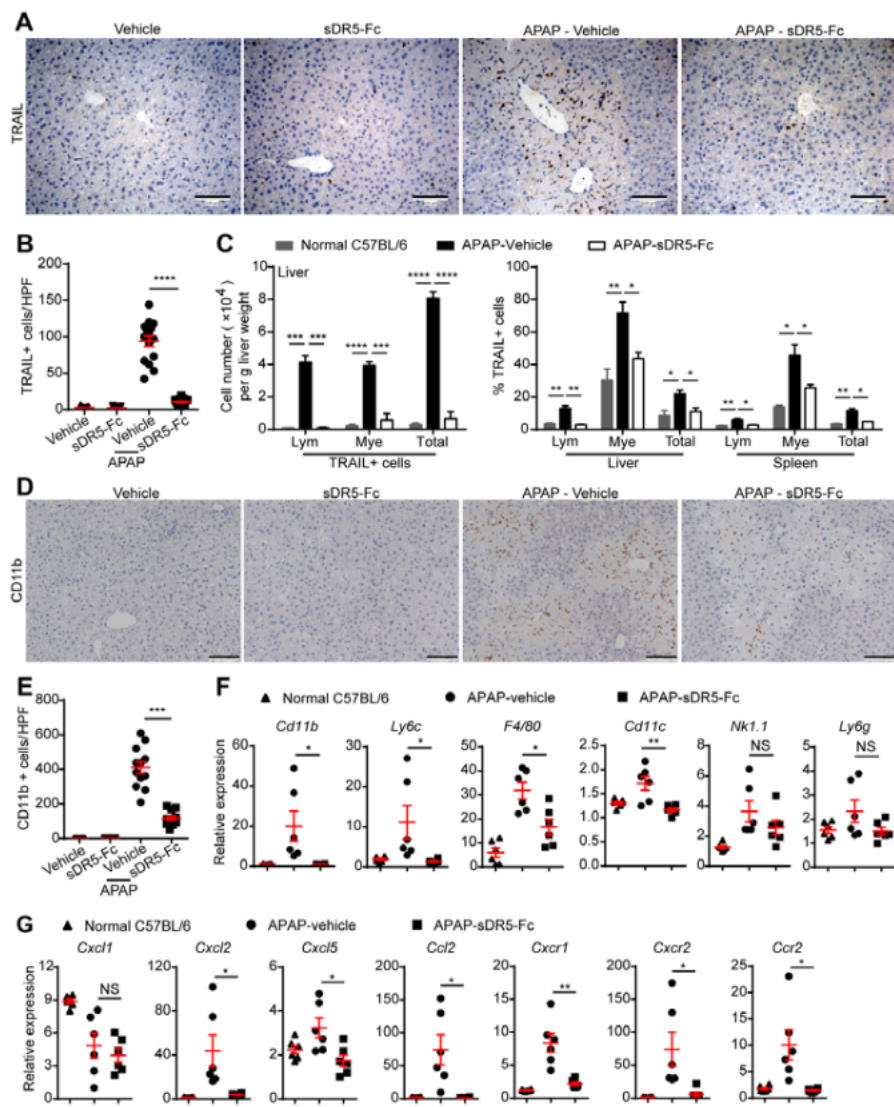


Figure 34: AS1501 attenuates APAP-induced leukocyte infiltration

Mice (n = 6) were administered APAP (300 mg/kg via intraperitoneal injection), followed by administration of either 9 mg/kg AS1501 (expressed as sDR5-Fc in the published article) or saline as control after 1 hour. Liver samples were collected at 24 hours post-APAP injection for histopathological analysis (A, D) and RNA expression analysis (F, G), or liver and spleen samples were collected at 8 hours post-APAP injection for flow cytometry analysis (C).

(A, D) Immunohistochemical staining of TRAIL+ and CD11b+ leukocytes in the liver. Scale bar: 100 μm.

(B, E) As shown in A and D, the number of TRAIL+ and CD11b+ leukocytes per high-power field (HPF) in liver sections.

(C) Number or percentage of TRAIL-positive leukocytes in the liver and spleen. Lym, lymphocytes; Mye, bone marrow cells.

(F, G) Quantitative qPCR analysis was performed to measure the mRNA expression levels of Cd11b, Ly6c, F4/80, Cd11c, Nk1.1, Ly6g, Cxcl1, Cxcl2, Cxcl5, Ccl2, Cxcr1, Cxcr2, and Ccr2 in the liver. The presented data are relative to gene expression levels. All values shown represent the mean $\pm$ SEM of representative experiments from three independent trials. Double-tailed unpaired t-tests were used; *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. NS indicates no significant difference (P > 0.05).

APAP induces severe inflammatory responses in the liver. By immunohistochemistry, we observed a concentration of large TRAIL⁺ cells in severely damaged liver tissues of mice treated with APAP (Figure 8A). Treatment with AS1501 (code sDR5-Fc) significantly reduced the number of these TRAIL⁺ cells (Figure 8B). Flow cytometry analysis further revealed that APAP markedly increased both the quantity and percentage of TRAIL⁺ leukocytes in the liver and spleen, an effect that was significantly attenuated by AS1501 (Figure 8C). Most TRAIL⁺ bone marrow cells are CD11b⁺, and we also observed abundant CD11b⁺ cells localized in severely damaged liver regions (Figure 8D); AS1501 significantly reduced the infiltration of these CD11b⁺ cells (Figure 8E). CD3⁺, CD4⁺, and CD8⁺ T cells were detected in the livers of APAP-treated mice, indicating that the majority of leukocytes infiltrating the liver lesions are not T cells.

Consistent with these findings, APAP significantly upregulated the liver mRNA levels of inflammatory cell markers, and AS1501 treatment markedly attenuated this effect (Cd11b:20.05 $\pm$ 8.94 vs. 1.14 $\pm$ 0.03; Ly6c:11.17 $\pm$ 4.76 vs. 1.59 $\pm$ 0.22; F4/80:31.78 $\pm$ 5.16 vs. 17.36 $\pm$ 2.84; Figure 8F). Notably, APAP increased the levels of dendritic cell marker Cd11c (1.7-fold), granulocyte marker Ly6g (2.3-fold), and Nk1.1 (3.0-fold). AS1501 treatment had no significant effect on Ly6g and Nk1.1 levels but reduced Cd11c levels. We observed that APAP significantly upregulated the levels of Cxcl2, Ccl2, Cxcr1, Cxcr2, and Ccr2 (Figure 8G), indicating CXCL2- and CCL2-dependent recruitment of circulating MDSCs to the liver, which was significantly normalized by AS1501 (Figure 8G).

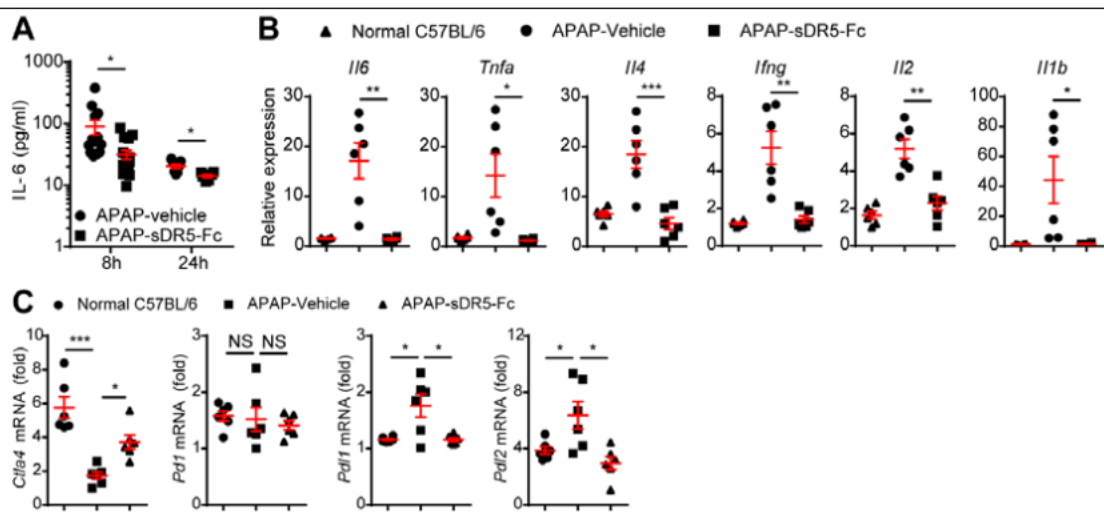


Figure 5: AS1501 4attenuates APAP-induced inflammatory response

Mice were treated with APAP (300 mg/kg, intraperitoneally) and then administered either 9 mg/kg of AS1501 or saline as a control group after 1 hour.

(A) Serum IL-6 levels were measured by cell counting bead array assay at 8 and 24 hours post APAP injection: n=17 at 8 hours, n=6 at 24 hours.

(B, C) Quantitative qPCR analysis was performed to measure the mRNA expression levels of Il6, Tnfa, Il4, Ifng, Il2, Il1b, Ctla4, Pd11, Pd12, and Pd1 in the liver. The presented data represent relative gene expression levels (n=6). All values are shown as mean $\pm$ SEM from representative experiments across three replicates. Double-tailed unpaired t-tests were used; *P<0.05, **P<0.01, ***P<0.001. NS indicates no significant difference (P>0.05).

Blockade of TRAIL via AS1501 significantly reduced serum IL-6 levels (Figure 9A), but no such effect was observed on serum TNF- α , IFN- γ , IL-10, IL-17A, IL-4, or IL-2, likely due to the low serum concentrations of these cytokines induced by APAP. However, we observed a significant reduction in the expression of cytokines such as IL-6, TNF- α , IFN- γ , IL-4, IL-2, and IL-1 β in the livers of mice treated with AD1501 compared to those receiving blank treatment after APAP overdose (Figure 9B).

Programmed death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) are key immune checkpoint receptors involved in inducing immune tolerance. We observed that APAP significantly reduced Ctla4 transcripts in the liver, while AS1501 reversed this effect (Figure 8C). However, APAP did not affect PD-1 mRNA levels. PD-1 has two ligands: PD-L1 and PD-L2. APAP increased the

expression of PD-L1 and PD-L2 mRNA, and AS1501 reversed their expression. These results demonstrate that AS1501 attenuates the inflammatory response induced by APAP.

The results of this study, when combined with in vitro experiments, further validated the mechanism of action of AS1501. AS1501 primarily inhibits hepatocyte apoptosis by blocking the TRAIL-DR5 pathway and additionally attenuates inflammatory responses.

6) Role of the TRAIL-DR5 signaling pathway in viral hepatitis models

To determine the potential role of TRAIL in HBV-induced hepatitis, Professor Youhai Chen's team in 2007 investigated the effects of HBV and its X protein (HBx) on TRAIL-induced hepatocyte apoptosis both in vivo and in vitro [40]. They found that hepatitis and hepatocyte death in HBV-transgenic mice were significantly inhibited by soluble DR5 receptors that block TRAIL function. Additionally, transfection of HBV or HBx into hepatocellular carcinoma cell lines markedly increased their sensitivity to TRAIL-induced apoptosis. This enhanced TRAIL sensitivity was associated with a significant upregulation of Bax protein expression. Knockdown of Bax expression using Bax-specific small interfering RNA (siRNA) blocked HBV-induced hepatitis and hepatocyte apoptosis. The degradation of cysteine proteases 3 and 9—rather than Bid or caspase-8—was predominantly affected by Bax knockdown. These results confirm that HBV renders hepatocytes susceptible to TRAIL-induced apoptosis via Bax, and that Bax-specific siRNA can be employed to suppress HBV-induced hepatocyte death.

➤ **Establishment of HBV animal models**

The hepatitis B virus (HBV) is the smallest known DNA virus in humans, with a genome size of approximately 3.2 kb and composed of partial double-stranded DNA. It belongs to the family Hepadextranidae and exhibits strict hepatotropism and species specificity, typically infecting liver tissues and causing acute or chronic hepatitis; under natural conditions, it can only infect humans and a few primates [4]. These characteristics impose significant limitations on the use of animal models for HBV research. Our laboratory has also attempted to establish an HBV animal model using mice, but the effort was abandoned due to unsuccessful gene transfer.

Table 1 HBV Animal Models and Their Advantages and Disadvantages

model	merit	shortcoming	remarks
Non-human primates	Highly susceptible to natural HBV infection; exhibits extreme similarity to human infection.	Constrained by ethical considerations; high cost; large size	Similar to human genes
Tree squirrel	Relatively small in size; capable of directly infecting HBV	Low infection efficiency; causes only transient, mild infections; exhibits	It shares a close evolutionary

		low viral load	origin with primates.
Ducks, groundhogs, etc.	It can be infected by HBV-related viruses; primary hepatocytes are highly susceptible.	The animal has a relatively large body size; its immune background is unclear; HBV-related viruses differ from HBV, and the obtained data may not be applicable to HBV.	as a host for HBV-related viruses
mouse	Small in size and easy to handle; inexpensive and readily available; well-defined genetic background; short breeding cycle with high litter size; numerous techniques and reagents are available for mouse genetic modification.	Mice lack HBV receptors and cannot be naturally infected; the transfection of HBV genomes requires advanced technical expertise and involves complex procedures; immunodeficient mice demand stringent breeding conditions.	Due to significant differences from human biology, the obtained results are not entirely applicable to humans.

2.2.2.2. toxicologic study

2.2.2.2.1. Single-dose toxicity study

➤ Toxicity study of AS1501 administered via single intravenous infusion in SD rats

In the toxicity study of AS1501 administered via single intravenous infusion in SD rats, 40 rats (20 per sex) were randomly divided into four groups (5 rats per sex per group): the negative control group (sodium chloride injection, 0 mg/kg), the low-dose test group (AS1501,375 mg/kg), the medium-dose test group (AS1501,750 mg/kg), and the high-dose test group (AS1501,1500 mg/kg). Administration was performed via tail vein infusion using an infusion pump at a volume of 15 mL/kg and a rate of approximately 5 mL/kg/min. Animals were monitored continuously for 4 hours post-administration. During the study, regular clinical observations, body weight measurements, and food intake were recorded. Upon completion of the observation period (Day 15), all animals were euthanized and subjected to gross anatomical examination.

The solutions of AS1501 prepared on Day 1 at concentrations of 25,50, and 100 mg/mL were analyzed, demonstrating an accuracy range of 101.04%–106.79%. The coefficient of variation (CV) for the upper, middle, and lower layers at each concentration ranged from 0.04% to 1.50%, indicating accurate preparation of AS150 liquid and suitability for testing. No AS1501 was detected in the negative control solution.

During the trial period, none of the animals exhibited death or near-death conditions. No abnormal changes related to the test substance were observed in clinical observations, body weight, or food intake across all administered animals. No abnormalities associated with the test substance were noted during gross anatomical examination of the animals.

In conclusion, under these experimental conditions, when AS1501 was administered via single intravenous infusion at doses of 375,750, and 1500 mg/kg to SD rats, the no observed adverse effect level (NOAEL) was determined to be 1500 mg/kg.

➤ **Toxicity study of AS1501 administered via single intravenous infusion in cynomolgus monkeys**

In the toxicity study of AS1501 administered via single intravenous infusion in cynomolgus monkeys, eight cynomolgus monkeys (four per sex) were randomly divided into four groups (one per sex per group): a negative control group (sodium chloride injection), a low-dose test substance group (300 mg/kg), a medium-dose test substance group (600 mg/kg), and a high-dose test substance group (1200 mg/kg). Administration was performed via subcutaneous intravenous infusion using an injection pump at the hind limbs, with a dosage volume of 12 mL/kg and a delivery rate of approximately 1 mL/kg/min. Animals were monitored continuously for 4 hours post-administration, during which clinical observations, body weight, body temperature, electrocardiogram (ECG), blood cell counts, coagulation function, blood biochemistry, and urine analysis were regularly assessed. Upon completion of the observation period (Day 16), all animals were euthanized and subjected to gross anatomical examination.

During the trial period, no deaths or near-death conditions were observed in any animals. No abnormalities related to the test substance were detected in clinical observations, body weight, body temperature, electrocardiogram (ECG), blood cell counts, coagulation function, blood biochemistry, or urine analysis in animals administered doses of 300,600, and 1200 mg/kg. Gross anatomical examination revealed no pathological changes associated with the test substance during animal necropsies.

In conclusion, under these experimental conditions, AS1501 administered via single intravenous infusion at doses of 300,600, and 1200 mg/kg in cynomolgus monkeys did not exhibit significant toxic effects, with the no observed adverse effect level (NOAEL) determined to be 1200 mg/kg.

Table 1 Toxicity profile of a single dose of AS1501

Species/Lineage	medication	Dosage (mg/kg)	Gender and Number in Each Group	Maximum non-lethal dose observed (mg/kg)	Approximate lethal dose (mg/kg)	end of test
SD rat	The injection pump was	0	Each group	1500	Not observed	1. During the trial period, none of the

	used for intravenous administration via the tail vein, with a dosage volume of 15 mL/kg and a infusion rate of 5 mL/kg/min.	375 750 1500	consists of 10 animals (5 males and 5 females).			animals exhibited signs of death or near-death conditions. 2. During the trial period, no abnormal changes related to the test substance were observed in the clinical manifestations, body weight, or food intake of the administered animals. 3. No abnormal changes related to the test sample were observed during gross anatomical examination of the animal.
Crayfish-eating monkey	The injection pump delivers the medication via tail vein infusion at a dosage of 5 mL/kg and a delivery rate of 5 mL/kg/min.	0 300 600 1200	Each group consists of 2 animals (1 male and 1 female).	1200	Not observed	1. During the trial period, none of the animals exhibited signs of death or near-death conditions. 2. During the trial period, no abnormal changes related to the test substance were observed in clinical observations, body weight, body temperature, electrocardiogram (ECG), blood cell counts, coagulation function, blood biochemical parameters, or urine analyses in animals across all measurement groups. 3. No pathological

						changes related to the test specimen were observed during gross examination of the animal anatomy.
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2.2.2.2.2. Repeated Dose Toxicity Study

- Dose exploration study: A 4-week toxicity trial involving repeated intravenous administration of AS1501 to SD rats
See Table 10 for details.
- Dose exploration study: A toxicity trial evaluating the repeated intravenous administration of AS1501 to cynomolgus monkeys over a 4-week period
See Table 10 for details.
- Toxicity study of repeated intravenous infusion of AS1501 in SD rats over 13 weeks followed by a 4-week recovery period

In the toxicity study of AS1501 administered by repeated intravenous injection in SD rats over a period of 13 weeks followed by a 4-week recovery period, the potential toxic effects and in vivo metabolism of AS1501 following weekly intravenous infusion for 13 consecutive weeks were evaluated, along with the resolution of toxic effects during the 4-week post-administration recovery period. A total of 192 SD rats (96 per sex) were randomly divided into 8 groups: Groups 1–4 underwent toxicology studies (15 rats per sex per group), while Groups 5 (6 rats per sex) and Groups 6–8 (10 rats per sex per group) underwent toxicokinetic studies. Sodium chloride injection was administered as a negative control to animals in Groups 1 and 5; AS1501 at doses of 20 mg/kg, 60 mg/kg, and 200 mg/kg was administered to animals in Groups 2 and 6, Groups 3 and 7, and Group 48, respectively. Intravenous administration was performed once weekly for 13 weeks, followed by a 4-week recovery period, with a dosing volume of 10 mL/kg and a infusion rate of approximately 3.33 mL/kg/min. During the study, clinical observations, body weight, food intake, body temperature, ophthalmic examinations, blood cell counts, coagulation function, blood biochemistry, urine analysis, T lymphocyte subpopulations, cytokines, serum complement levels, and serum antibody titers were monitored. Following the completion of the administration period, the first 10 animals per sex per group (excluding animal 1725377 in Group 1) were euthanized on day D93 as scheduled; the remaining 5 animals per sex per

group were euthanized at the end of the 4-week recovery period (D120) as planned. All animals underwent gross anatomy examination, organ weight measurement, and histopathological analysis.

The test solution with concentrations of 2, 6, and 20 mg/mL for D1, D5, and D92 was analyzed. The accuracy range of the prepared AS1501 solutions at each concentration was 96.00%–105.54%, with coefficient of variation (CV%) values for the upper, middle, and lower layers ranging from 0.03% to 2.04%, indicating accurate preparation of the AS1501 solution and compliance with requirements. No AS1501 was detected in the negative control sample.

During this study, one male animal in the negative control group (Animal No.: 1725377) died unexpectedly on day D64. Prior to death, it exhibited mild hunchback, pallor of the ears and limbs, and grossly enlarged liver and spleen. Histopathological examination revealed myeloid leukemia of bone marrow origin with metastases in the liver, spleen, and lungs. Therefore, the cause of death was determined to be multi-organ failure. Throughout the trial, clinical observations, body weight, food intake, body temperature, ophthalmic examinations, blood cell counts, coagulation function, blood biochemistry, urine analysis, T lymphocyte subpopulations, cytokines, and serum complement levels in animals administered the test substances at doses of 20,60, and 200 mg/kg showed no abnormalities associated with the test substances.

SD rats were administered AS1501 at doses of 20,60, and 200 mg/kg once weekly for a continuous period of 13 weeks. Both the plasma peak concentration (C_{max}) and plasma exposure of AS1501 showed positive correlations with the dose administered on day 1 (D1) and day 85 (D85) after the first administration. No significant gender differences were observed in the metabolic characteristics of AS1501 in animals. Following continuous administration, the accumulation factors (AUC_{last}, 13 administrations/AUC_{last}, first administration) were 3.18,3.78, and 2.55 in male animals, respectively, compared to 2.73,2.15, and 1.77 in female animals. The specific toxicokinetic parameters are presented as follows:

Table 1 Toxicokinetic parameters of repeated intravenous infusion of AS1501 in SD rats over 13 weeks

period	group mg/kg	sex	T _{max} h	C _{max} mg/mL	SE_C _{max} mg/mL	AUC _{last} h*mg/mL	SE_AUC _{last} h*mg /mL	AUC _{inf} h*mg/mL
D1	Low-dose	M	0.05	0.42	0.01	15.76	0.33	24.23
	group							
	20	F	0.05	0.40	0.02	14.82	0.41	24.35
	Medium-	M	0.05	1.08	0.02	37.44	0.92	53.22

dose group								
	60	F	0.05	1.07	0.04	44.82	0.82	61.22
D85	High-dose group	M	0.05	3.64	0.09	129.86	2.82	209.42
	200	F	0.05	3.65	0.25	142.77	1.08	235.77
	Low-dose group	M	0.05	0.77	0.04	50.09	2.42	98.6
	20	F	0.05	0.63	0.03	40.39	1.04	82.4
	Medium-dose group	M	0.05	1.98	0.08	141.59	3.78	293.11
	60	F	0.05	1.74	0.08	96.15	5.07	182.08
	High-dose group	M	0.05	6.25	0.25	331.68	10.27	671.68
	200	F	0.05	5.43	0.24	252.61	11.56	492.18

During the trial, no pathological changes associated with the test substance were observed in the organ weights, gross anatomy, or histopathological examinations of SD rats subjected to euthanasia at the end of drug administration or at the conclusion of the recovery period.

In conclusion, AS1501 was administered via repeated intravenous infusion at doses of 20,60, and 200 mg/kg to SD rats once weekly for 13 consecutive weeks. Under these experimental conditions, no significant toxic effects were observed, with the no observed adverse effect level (NOAEL) determined at 200 mg/kg. At this dose, the mean plasma concentration (Cmax) and area under the concentration-time curve (AUClast) of AS1501 were 6.25 mg/mL and 331.68 h·mg/mL in male animals, respectively; whereas in female animals, they were 5.43 mg/mL and 252.61 h·mg/mL, respectively.

➤ **AS1501 toxicity study involving repeated intravenous administration to cynomolgus monkeys for 13 weeks followed by a 4-week recovery period**

In the toxicity study of AS1501 administered intravenously repeatedly to cynomolgus monkeys over 13 weeks followed by a 4-week recovery period, the potential toxic effects and in vivo metabolism of AS1501 following repeated intravenous infusion (once weekly for 13 weeks) were evaluated, along with the resolution of toxic effects and possible delayed toxicity during the 4-week post-administration period. Forty cynomolgus monkeys (20 per sex) were randomly divided into four groups (5 per sex per group): a negative control group (sodium chloride injection), a low-dose test group (10 mg/kg), a medium-dose test group (30 mg/kg), and a high-dose test group (100 mg/kg). Administration was performed via subcutaneous intravenous infusion into the posterior limb veins once weekly for 13 weeks, followed by a 4-week recovery period. During the study, clinical observations were conducted, and

parameters including body weight, food intake, body temperature, electrocardiographic parameters, blood pressure, blood oxygen saturation, ocular examination, hematologic counts, coagulation function, blood biochemistry, urine analysis, lymphocyte subsets (CD3+, CD3+/CD4+, CD3+/CD8+, CD4+/CD8+ ratio), cytokines (TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-6), serum complement levels (C3 and C4), and serum anti-drug antibodies were measured. The first three animals per group and per sex were euthanized on day D93, and the remaining two animals per group and per sex were euthanized on day D120. All animals underwent gross dissection, organ weighing, and histopathological examination.

The AS1501 solutions prepared at concentrations of 2, 6, and 20 mg/mL on days 1, 50, and 92 were analyzed, demonstrating an accuracy range of 95.27%–104.47%. The coefficient of variation (CV) for the upper, middle, and lower layers at each concentration ranged from 0.02% to 1.79%, indicating accurate preparation of the AS1501 solution suitable for experimental use. No AS1501 was detected in the negative control group.

During the trial period, no deaths or near-death cases were observed in any animals across Groups 1 to 4.

During the trial period, no abnormalities related to the test substance were observed in animals administered the 10,30, or 100 mg/kg doses regarding clinical manifestations, body weight, food intake, body temperature, electrocardiographic parameters, blood pressure, blood oxygen saturation, ophthalmic examinations, blood cell counts, coagulation function, blood biochemistry, urine analysis, lymphocyte subpopulations, cytokines, or serum complement levels.

Crab-eating macaques were administered AS1501 at doses of 10,30, and 100 mg/kg once weekly for a continuous period of 13 weeks. Both the plasma peak concentration (C_{max}) and plasma exposure of AS1501 showed positive correlations with the dosing dose at both the first administration (D1) and the 12th administration (D85). No significant gender differences were observed in the metabolic characteristics of AS1501 in animals. After 12 weeks of weekly administration in low-, medium-, and high-dose groups, the accumulation factors (AUC_{last}, 12 administrations vs. AUC_{last}, first administration) were 2.11,2.11, and 1.71, respectively, indicating no significant accumulation of AS1501 in animals. The specific toxicokinetic parameters are as follows:

Table 1 Toxicokinetic parameters of AS1501 following repeated intravenous infusion in crab-eating macaques over 13 weeks

period	group Dosage (mg/kg)	C _{max} mg/mL	AUC _{last} h*mg/mL	AF
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First admini stratio n	Low-dose group (n=10)		Mean	0.21	11.38	
	10		SD	0.04	1.12	
	Middle-dose group (n=10)		Mean	0.72	37.28	
	30		SD	0.07	5.10	
	High-dose group (n=10)		Mean	2.15	122.91	
	100		SD	0.41	12.17	
13 times dose	Low-dose group (n=10)		Mean	0.35	23.03	2.11
	10		SD	0.06	6.85	0.14
	Middle-dose group (n=10)		Mean	1.12	77.37	2.11
	30		SD	0.14	12.50	0.23
	High-dose group (n=10)		Mean	3.12	210.94	1.71
	100		SD	0.39	38.63	0.24

During the trial period, anti-drug antibodies were detected in one animal each from the negative control group and the 30 mg/kg and 100 mg/kg dose groups on days D93, D120, and D-1 (confirmed positive), with titers all <1. In the negative control group, one female animal (1725249) exhibited detectable anti-drug antibodies on days D93 and D120 (confirmed positive), with titers of 1:4 and 1:2, respectively. In the 10 mg/kg dose group, one male animal (1725253) showed detectable anti-drug antibodies on days D29, D50, and D64 (confirmed positive), with titers of <1, 1:2, and 1:32, respectively. No anti-drug antibodies were detected in the remaining animals during the trial.

After administration of euthanasia or recovery-phase euthanasia, no test substance-related pathological changes were observed in the organ weights, gross anatomical examinations, or histopathological analyses of animals in Groups 1–4.

In conclusion, when AS1501 was administered via repeated intravenous infusion at doses of 10,30, and 100 mg/kg to rhesus monkeys once weekly for 13 consecutive weeks, no toxicity related to the test substance was observed in the animals. Under these experimental conditions, the no observable adverse effect level (NOAEL) of AS1501 following repeated intravenous administration in rhesus monkeys was determined to be 100 mg/kg. At the 100 mg/kg dose, the peak plasma concentration (Cmax) and area under the concentration-time curve (AUClast) of AS1501 in animals after the 13th administration (D85) were 3.12 mg/mL and 210.94 h·mg/mL, respectively.

Table 1 Summary of the 4-week repeated-dose toxicology study for AS1501

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
rat , SD	Intravenous infusion	4 weeks	Negative control	5	yes	1. Animal mortality and near-death conditions

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
			group: 0 mg/kg			During the trial period, no deaths or near-death events related to the test substance were observed in any group of animals. One male animal (Animal No.16-8895) in the 20 mg/kg dose group was found dead on D15 (third administration). No abnormal clinical symptoms were observed prior to death, and gross anatomical examination revealed no abnormalities in major tissues or organs. Based on these findings, it is inferred that the animal's death likely resulted from asphyxia caused by improper restraint during administration and was unrelated to the test substance. 2. clinical observation During the trial period, no abnormalities were observed in the clinical examinations of animals across all groups. 3. weight During the trial period, no statistically significant changes in body weight or weight gain were observed in either male or female animals at the 20,60, and 200 mg/kg dose groups compared to the control group receiving the same excipient. 4. capacity for eating During the trial period, the food intake of animals in the treatment group was similar to that of the control group receiving auxiliary agents, with no significant difference observed. 5. hematology Compared with the control group receiving excipients, no statistically significant changes in blood cell counts were observed in either male or female animals at the 20,60, and 200 mg/kg dose groups. 6. Blood Biochemistry Compared with the control group receiving excipients, no
			Low-dose group: 20 mg/kg	5		
			Middle-dose group: 60 mg/kg	5		
			High-dose group: 200 mg/kg	5		

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						statistically significant changes were observed in blood biochemical parameters among male and female animals at the 20,60, and 200 mg/kg dose groups. 7. General anatomical examination No pathological changes were observed during gross examination of the animals.
Crayfish-eating monkey	Intravenous infusion	4 weeks	Negative control group: 0 mg/kg	1	yes	1. Animal mortality and near-death conditions During the trial period, no deaths or near-death cases were observed in any group of animals. 2. clinical observation duration of test , Group 1 (Excipient control group): One female animal exhibited sparse body hair from D-1 to D-14; small amounts of soft feces were observed on D-14 and D-15. In Group 2 (10 mg/kg), one male animal (15-8923) exhibited moderate yellow loose stools on day D4, followed by small amounts of soft stools on day D6. One female animal (15-8924) developed a moderate wound on the left forelimb from days D7 to D30; she showed small amounts of yellow loose stools on day D9, small amounts of soft stools on day D19, moderate amounts of yellow loose stools on day D20, and small amounts of brown loose stools on day D21. No significant abnormal clinical symptoms were observed in animals receiving Group 3 (30 mg/kg). In Group 4 (100 mg/kg), one male animal (ID 15-8927) exhibited small amounts of yellow loose stool from D-1 to D-3, and a mild wound was observed on the right forelimb from D-15 to D-21.
			Low-dose group: 10 mg/kg	1		
			Middle-dose group: 30 mg/kg	1		
			High-dose group: 100 mg/kg	1		

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						In animals 15 – 8923, self-biting behavior was observed during administration on days 8, 15, and 22 (with both hind limbs restrained during administration); in animal 15–8927, self-biting behavior was observed during administration on day 15 (with both hind limbs restrained), resulting in wounds to the forelimbs of both animals. Due to the limited number of animals, a correlation between this phenomenon and the test substance could not be established. The symptoms of soft stools, loose stools, or sparse coat observed in the aforementioned animal groups were considered unrelated to administration, as no significant dose-response relationship was observed and these manifestations could also occur prior to drug administration. 3. weight During the trial period, no significant abnormal changes in body weight were observed in any group of animals. 4. temperature During the trial period, the body temperatures of animals in the test group fluctuated within the normal reference range, with no significant abnormalities associated with the test substance observed. 5. electrocardiogram During the trial period, no significant abnormalities related to the test substance were observed in the electrocardiogram waveforms, heart rate, P-R interval, QRS duration, or QT interval of animals in the test group. 6. hematology During the trial period, no significant abnormalities related to the test substance were observed in

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						the blood cell counts of animals in the test group. 7. coagulation function During the trial period, no significant abnormalities related to the test substance were observed in the coagulation parameters of animals in the test group. 8. Blood Biochemistry During the trial period, no significant abnormalities related to the test substance were observed in the biochemical parameters of blood samples from animals in the test group. 9. URAN During the trial period, no significant abnormalities related to the test substance were observed in the urine parameters of animals in the test group. 10. Organ weight During the trial period, no significant abnormalities related to the test substance were observed in the organ weights, organ-to-body ratio, or organ-to-brain ratio of animals in the test group. 11. General observation No abnormal changes were observed in the gross examination of animals euthanized after drug administration.

Table 1 Summary of toxicological studies for AS1501 repeated administration over 13 weeks with a 4-week recovery period

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
rat , SD	Intravenous infusion	13 weeks	<u>Toxicology Studies</u>		yes	1. Animal mortality and near-death conditions During this study, one female animal in the toxicology control group (Animal No.: 17-25377) was found dead on day D64. Prior to death, it exhibited mild hunchback, pallor of the left ear and limbs, and grossly demonstrated hepatomegaly
			Negative control group: 0 mg/kg	10+5		
			Low-dose group: 20 mg/kg	10+5		

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
			Middle-dose group: 60 mg/kg	10+5		and splenomegaly. Histopathological examination revealed bone marrow-derived myeloid leukemia with metastases in the liver, spleen, and lungs. Therefore, the cause of death was determined to be multi-organ failure.
			High-dose group: 200 mg/kg	10+5		
			<u>Toxicology Studies</u>			2. clinical observation
			Negative control group: 0 mg/kg	4+2		During the trial period, no abnormal changes related to the test substance were observed in the clinical examinations of all animals.
			Low-dose group: 20 mg/kg	8+2		During the trial period, all observed abnormal clinical symptoms in the animal groups were sporadic or occasional. Due to their low incidence and absence of a dose-response relationship, these findings were deemed unrelated to the test substance.
			Middle-dose group: 60 mg/kg	8+2		
			High-dose group: 200 mg/kg	8+2		3. weight
						During the trial period, no abnormal changes in body weight or weight gain were observed in all animals related to the test substance. Compared with the negative control group during the same period, mild but statistically significant changes in weight gain were noted ($P \leq 0.05$), including: elevated W12 and W11 values in male animals; elevated W12 and reduced W14 values in male animals; elevated W1 and reduced W16 values in female animals; as well as elevated W12 and elevated W11 values in male animals at the 200 mg/kg dose group. These changes, characterized by small fluctuations within the normal reference range for SD rats, were deemed unrelated to the test substance.
						4. capacity for eating
						During the trial period, no abnormal changes in food intake were observed in the animals compared to the test substance.

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						5. temperature During the trial period, no abnormal changes in body temperature were observed in all animals associated with the test substance. Compared to the negative control group during the same period, mild but statistically significant changes in body temperature were noted ($P \leq 0.05$), including elevated body temperature in male animals and decreased body temperature in female animals at the D-1 time point in the 200 mg/kg dose group. These changes occurred prior to the initial administration and fluctuated within the normal reference range for SD rats; thus, they were considered unrelated to the test substance. 6. ophthalmic testing During the trial period, no abnormal changes were observed in the ophthalmic examinations of the animals. 7. blood count During the trial period, no abnormal changes in blood cell counts were observed in all animals associated with the test substance. Compared to the corresponding negative control group, mild but statistically significant alterations in blood cell counts were noted ($P \leq 0.05$), including: in female animals at the 20 mg/kg dose group, an increase in Mono (%) on day D93 and a decrease in red blood cell (RBC) count on day D120; in male animals at the 60 mg/kg dose group, an increase in Eos (%) on day D93, a decrease in RBC count and an increase in MCH on day D120; and in female animals at the 200 mg/kg dose group, a decrease in RBC count and an increase in MCH on day D120. These changes were

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						considered unrelated to the test substance due to their small magnitude and within the normal reference range for SD rats. 8. coagulation function During the trial period, no abnormal changes in coagulation function were observed in any of the animals associated with the test substance. 9. Blood Biochemistry During the trial period, no abnormal biochemical changes related to the test substance were observed in the blood profiles of all animals. Compared with the negative control group during the same period, mild but statistically significant alterations were noted in the animal blood biochemistry ($P \leq 0.05$), including elevated Glu levels in female animals at the 20 mg/kg dose group on day D120 and decreased urea levels in female animals at the 60 mg/kg dose group on day D120. These changes were considered unrelated to the test substance due to their small magnitude and within the normal reference ranges for SD rats. 10. URAN During the trial period, no abnormal changes related to the test substance were observed in the animal urine analyses. 11. Lymphocyte subsets During the trial period, no abnormal changes related to the test substance were observed in the lymphocyte subsets of all animals. 12. cell factor During the trial period, no abnormal changes in the animals' cytokine levels were observed related to the test substance. 13. Serum complement During the trial period, no abnormal changes in the animals' cytokine

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						levels were observed related to the test substance. 14. Drug-resistant antibodies During the trial period, no anti-AS1501 antibodies were detected in the animals. 15. Toxicokinetic Analysis No significant gender differences were observed in the pharmacokinetic parameters (Cmax and AUClast) among animals across all groups after the initial administration and the 12th dose. The dose ratios among animals in the low-, medium-, and high-dose groups (20,60, and 200 mg/kg) were 1:3:10, respectively. Both Cmax and AUClast values showed positive correlations with the administered doses. Following weekly administration for 12 consecutive doses, the plasma drug accumulation factors (AUClast at 12 doses vs. AUClast at the first dose) were 3.18,3.78, and 2.55 in male animals, and 2.73,2.15, and 1.77 in female animals. 16. Organ weight Compared with the negative control group during the same period, statistically significant changes were observed in organ weight, visceral-to-brain ratio, and visceral-to-brain volume ratio ($P \leq 0.05$) in the animals. Specifically, in the male animals of the 200 mg/kg dose group, testicular weight and visceral-to-brain ratio decreased on day 93. Due to the small magnitude of these fluctuations and the absence of abnormal histopathological findings, this change was deemed unrelated to the test substance. 17. General anatomical observation During this study, no pathological changes associated with the test substance were observed in SD rats

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						undergoing euthanasia at the end of drug administration or at the conclusion of the recovery period. Histopathological examination revealed renal lesions (renal cell carcinoma) in animals 17–25465 from the high-dose group; renal lesions (renal cell carcinoma) in animals 17–25480; and a breast mass (adenocarcinoma) in animal 17–25479 during euthanasia at the recovery period. These pathological findings were attributed to spontaneous neoplastic changes in SD rats and were not related to the test substance. 18. Histopathological examination During this study, histopathological examinations of SD rats euthanized at the end of drug administration and at the conclusion of the recovery period revealed no pathological changes associated with the test substance. However, these rats exhibited local injection reactions characterized by vascular degeneration and edema, thickening of the intima or entire vascular wall, vascular wall necrosis or fibrosis, fibrosis of the adventitia and/or perivascular tissue, thrombosis, inflammatory cell infiltration in the perivascular region, and/or hemorrhage. These pathological changes were attributed to the intravenous injection procedure.
Crayfish-eating monkey	Intravenous infusion	13 weeks	Negative control group: 0 mg/kg	3+2	yes	1. Animal mortality and near-death conditions During the trial period, no deaths or near-death cases were observed in any group of animals. 2. clinical observation During the trial period, no abnormal changes related to the test substance were observed in the clinical examination of all animals. Additionally, some sporadic or

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						occasional symptoms were noted in the animals, including loose stools, sparse dorsal coat, wounds, and scabs. These symptoms were considered unrelated to the test substance due to their mild severity and low incidence. 3. weight During the trial period, no abnormal changes in body weight or weight gain were observed in all animals related to the test substance. Compared with the negative control group during the same period, mild but statistically significant changes in body weight gain were noted ($P \leq 0.05$), including reduced weight gain in female animals at the 30 mg/kg dose group and reduced weight gain in female animals at the 100 mg/kg dose group. These changes, characterized by small fluctuations within the normal reference range for rhesus monkeys, were deemed unrelated to the test substance. 4. capacity for eating During the trial period, no abnormal changes in food intake were observed in the animals. 5. temperature During the trial period, no abnormal changes in body temperature were observed in all animals associated with the test substance. 6. electrocardiogram During the trial period, no abnormal changes related to the test substance were observed in the electrocardiographic parameters of all animals. Compared with the negative control group during the same period, mild but statistically significant alterations were noted in the electrocardiographic parameters ($P \leq 0.05$), including: reduced heart rate and prolonged Q-T interval in

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						male animals at doses of 2 mg/kg on days D1, D57, and D85; decreased heart rate and shortened QRS duration on day D1 and D57 in the 10 mg/kg dose group; reduced heart rate and shortened QRS duration on days D1, D57, and D85 in the 50 mg/kg dose group. These changes, characterized by small amplitude fluctuations within the normal reference range for rhesus monkeys, were deemed unrelated to the test substance. 7. blood pressure During the trial period, no abnormal changes in blood pressure were observed in all animals associated with the test substance. 8. degree of blood oxygen saturation During the trial period, no abnormal changes in blood oxygen saturation were observed in all animals associated with the test substance. 9. ophthalmic testing During the trial period, no abnormal changes related to the test substance were observed in the ophthalmic examinations of the animals. One female animal (1725270) in the 30 mg/kg dose group exhibited partial eyelid tissue defects on days D-1, D90, and D119. As this symptom occurred prior to the first administration, it was deemed unrelated to the test substance. 10. blood count During the trial period, no abnormal changes in blood cell counts were observed in all animals associated with the test substance. Compared to the corresponding negative control group, mild but statistically significant alterations in blood cell counts were noted ($P \leq 0.05$), including: elevated Baso ($\times 10^9/L$) and LUC ($\times 10^9/L$) levels in male animals at the 10 mg/kg dose group

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						on day D2; decreased Baso ($\times 10^9$ /L) levels in female animals at the 100 mg/kg dose group on day D-1; reduced hemoglobin (HGB) and red blood cell specific volume (HCT) levels on days D44; and decreased HGB, HCT, and mean corpuscular volume (MCV) levels on day D93. These changes were considered unrelated to the test substance due to their minor fluctuations within the normal reference ranges for rhesus monkeys. 11. coagulation function During the trial period, no abnormal changes in coagulation function were observed in all animals related to the test substance. Compared with the negative control group during the same period, mild but statistically significant alterations in coagulation function were noted ($P \leq 0.05$), including a reduction in APTT in male animals at the 10 mg/kg dose of the test substance on day D93. These changes were considered unrelated to the test substance due to their small magnitude and within the normal reference range for rhesus monkeys. 12. Blood Biochemistry During the trial period, no abnormal biochemical changes related to the test substance were observed in the blood profiles of all animals. Compared with the negative control group during the same period, mild but statistically significant alterations were noted in the blood biochemistry of the animals ($P \leq 0.05$), including: elevated levels of alkaline phosphatase (ALP), γ - glutamyl transferase (GGT), and K⁺ in male animals at the 10 mg/kg dose group on day D-1; increased

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						ALP and GGT levels on day D-2; elevated ALP levels on day D-44; decreased plasma phosphate (P) levels in female animals on day D-1; elevated chloride (Cl-) levels in male animals at the 30 mg/kg dose group on day D-44; decreased P levels in female animals on days D-1 and D-2; elevated GGT levels in male animals on days D-1 and D-2; and reduced albumin (ALB) levels along with altered A/G ratios in female animals on days D-1 and D-93. These findings were considered unrelated to the test substance due to their minor fluctuations within the normal reference ranges for cynomolgus monkeys. 13. URAN During the trial period, no abnormal changes were observed in the urine analysis of the animals. 14. Lymphocyte subsets During the trial period, no abnormal changes related to the test substance were observed in the lymphocyte subsets of all animals. 15. cell factor During the trial period, no abnormal changes in cytokines were observed in the animals associated with the test substance. In one male animal (1725272) from the 100 mg/kg dose group of the test substance, elevated levels of cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-6) were detected on day 93; however, such elevations were not observed in other animals in the high-dose groups, indicating they were isolated incidents unrelated to the test substance. 16. Serum complement During the trial period, no abnormal changes in animal serum complement were observed associated with the test substance. Compared with the corresponding

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						negative control group, mild but statistically significant alterations were noted in animal serum complement levels ($P \leq 0.05$), including decreased serum complement C4 levels in male animals at doses of 10 mg/kg for both D-1 and D-2 days; decreased levels in male animals at 30 mg/kg for both D-1 and D-2 days; and a decrease in serum complement C4 levels in male animals at 100 mg/kg for D-2 day. These changes, characterized by small fluctuations within the normal reference range for rhesus monkeys, were deemed unrelated to the test substance. 17. Drug-resistant antibodies During the trial, anti-drug antibodies were detected in one animal each from the negative control group and the 30 mg/kg and 100 mg/kg dose groups on days D93, D120, and D-1 (confirmed positive), with titers all <1; one female animal (1725249) in the negative control group exhibited detectable anti-drug antibodies on days D93 and D120 (confirmed positive), with titers of 1:4 and 1:2, respectively. One male animal (1725253) in the 10 mg/kg dose group showed detectable anti-drug antibodies on days D29, D50, and D64 (confirmed positive), with titers of <1, 1:2, and 1:32, respectively. No anti-drug antibodies were detected in the remaining animals during the trial. Due to the low antibody titers or the occurrence in the negative control group, these findings are considered to have limited toxicological significance. 18. Pharmacokinetics The test substance was administered via weekly intravenous infusion to cynomolgus

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						monkeys. Following the initial administration, the Cmax values in male animals of the low-dose group were slightly higher than those in female animals (P <0.05; however, the difference was not significant, with a ratio of (male/female) = 1.37). No significant gender-related differences were observed in other groups. After the 12th administration, no notable differences in toxicokinetic parameters were noted between genders across all groups. The dose ratios among animals in the low-, medium-, and high-dose groups (10,30, and 100 mg/kg) of the test substance were 1:3:10. Following the first administration, the ratios of mean Cmax and AUClast values among these groups were 1:3.35:10.01 and 1:3.28:10.80, respectively; after the 13th administration, the corresponding ratios of mean plasma drug Cmax and AUClast values were 1:3.2:9.74 and 1:3.36:9.16, respectively. These results demonstrate that both plasma peak drug concentrations and plasma drug exposure are positively correlated with the administered doses. Animals in the low-, medium-, and high-dose groups of the test substance were administered once weekly for 13 consecutive weeks, resulting in cumulative area under the curve (AUClast; 12 administrations vs. AUClast at the first administration) values of 2.11,2.11, and 1.71, respectively. 19. Organ weight During the trial period, no abnormal changes related to the test substance were observed in the organ weights, visceral-to-body ratio, or visceral-to-brain ratio of all animals.

Species/Lineage	medication	Duration of administration	dosage	Number of animals/Gender	GLP	end of test
						20. General observation Euthanasia was performed on the day following drug administration (D93) and four weeks after discontinuation (D120). Gross anatomical examination of the animals revealed no abnormal changes associated with the test substance. 21. Histopathological examination Euthanasia was performed on the day following drug administration (D93) and four weeks after discontinuation (D120). Histopathological examinations of the animals revealed no abnormal changes associated with the test substance.

2.2.2.2.3. Local tolerance study

1) In vitro hemolytic test of AS1501 for injection on human red blood cells

In the in vitro hemolytic test of AS1501 for injection on human red blood cells, a concentration of 10 mg/mL was used as the test solution. Different volumes (0.5–0.1 mL) of the test solution and different volumes (2.5–2.9 mL) of 0.9% sodium chloride injection were added separately to glass test tubes containing 2.0 mL of 2% human red blood cell suspension. Concurrently, 3.0 mL of 0.9% sodium chloride injection and 3.0 mL of sterile water for injection served as negative and positive controls, respectively. The total volume of each test tube was 5.0 mL, and the samples were incubated at 37°C for 3 hours.

On the day of testing, concentration and homogeneity analyses were performed on the test solution. For the solution with a theoretical concentration of 10 mg/mL, the measured concentrations in the upper, middle, and lower layers were 10.39808 mg/mL, 10.39329 mg/mL, and 10.39508 mg/mL, respectively, yielding accuracy values of 103.98%,103.93%, and 103.95%. The coefficient of variation for concentrations in these three layers was 0.02%. No traces of the test substance were detected in either the negative or positive controls, and the analytical results complied with protocol requirements.

The negative control tube showed no hemolysis or agglutination, whereas the positive control tube exhibited complete hemolysis. All test tubes demonstrated partial hemolysis without agglutination. Under these experimental conditions, AS1501 for injection at a concentration of 10 mg/mL induced partial hemolysis of human red blood cells in vitro without causing agglutination.

Although AS1501 exhibited partial hemolysis in in vitro hemolytic assays using human red blood cells, repeated dosing toxicity studies in SD rats and cynomolgus monkeys revealed no abnormal changes in red blood cell count, nor were any test substance-related abnormalities observed on gross anatomical examination. Therefore, AS1501 is determined to be non-hemolytic.

2) Active systemic allergy test in guinea pigs using AS1501 for injection

In the active systemic allergy test using AS1501 for injection in guinea pigs, 34 animals (30 females and 3 males; 2 males for a supplementary trial) were randomly divided into three groups: negative control group, positive control group, and low-and high-dose test group, with 8 animals in each group (equal numbers of males and females). The negative control group (Group 1) received sodium chloride injection; the positive control group (Group 2) was administered human serum albumin at an sensitizing dose of 40 mg/kg and an inducing dose of 80 mg/kg; the low-dose test group (Group 3) and high-dose test group (Group 4) received AS1501 for injection. The sensitizing doses for the low-dose group were 1 mg/kg and 2 mg/kg, respectively, while those for the high-dose group were 3 mg/kg and 6 mg/kg. Sensitization was achieved via intraperitoneal injection administered every other day over three doses. On day 19 (D19), following the final sensitization, the first two animals per sex in each group were injected intravenously into the foot vein to induce an allergic reaction. No abnormal responses were observed in the two animals in the supplementary trial; therefore, additional induction was performed on the remaining animals in each group on D19.

Prior to stimulation, clinical observations in all groups 1–4 revealed no abnormal reactions. Following venous stimulation of the feet, none of the 8 animals in the negative control group exhibited allergic symptoms, indicating a negative allergic response; whereas the 8 animals in the positive control group displayed varying degrees of allergic reactions, including restlessness, hair standing, nose scratching, coughing, tachypnea, urination, dyspnea, purpura, gait instability, spasms, and Cheyne-Stokes respiration, with one animal deceased, demonstrating allergic responses ranging from weakly positive to extremely strong positive. In the low-dose group of the test substance, 8 animals exhibited similar allergic symptoms (hair standing, nose scratching, coughing, tachypnea, urination, dyspnea,

purpura, gait instability, spasms, Cheyne-Stokes respiration), with 5 animals deceased, showing positive to extremely strong allergic responses; in the high-dose group, 8 animals displayed severe allergic reactions (restlessness, hair standing, nose scratching, sneezing, coughing, tachypnea, urination, dyspnea, purpura, gait instability, spasms, Cheyne-Stokes respiration), with 4 animals deceased, exhibiting strong to extremely strong allergic responses. Under these experimental conditions, intraperitoneal administration of AS1501 at doses of 1 mg/kg and 3 mg/kg administered three times for sensitization, followed by intravenous stimulation at doses of 2 mg/kg and 6 mg/kg, resulted in the development of immediate hypersensitivity reactions in guinea pigs 14 days after the final sensitization.

The test substance AS1501 in this study is a humanized protein and constitutes a heterologous protein for guinea pigs. Given that guinea pigs are animals prone to immediate-type allergic reactions and exhibit high sensitivity to allergens, they are highly susceptible to allergic responses. Allergic reactions observed in animal studies do not necessarily translate directly to humans.

Although the active systemic allergy test in guinea pigs with AS1501 for injection demonstrated allergic reactions, repeated dosing toxicity studies in SD rats and cynomolgus monkeys revealed no anatomical abnormalities associated with the test substance, nor were any histopathological abnormalities observed. Therefore, AS1501 for injection is determined to be non-sensitizing.

3) Local irritancy

AS1501 is formulated as a lyophilized powder for injection and is administered intravenously in clinical practice. We conducted long-term toxicity studies involving SD rats and cynomolgus monkeys, including 13 weeks of repeated dosing followed by a 4-week recovery period, to evaluate its local irritant effects. During the rat study, SD rats euthanized at the end of dosing and at the conclusion of the recovery period exhibited local injection-related reactions, including vascular degeneration and edema, thickening of the intima or entire vascular wall, vascular wall necrosis or fibrosis, fibrosis of the adventitia and/or perivascular tissue, thrombosis, inflammatory cell infiltration and/or hemorrhage in the perivascular region. These pathological changes were attributed to the intravenous administration procedure. No local adverse effects of AS1501 were observed in the cynomolgus monkey study. The injection sites were also meticulously examined histopathologically. Histopathological analyses performed on animals from groups 1 to 4 at day 93 (end of dosing) and day 120 (4 weeks post-dose cessation) revealed no test substance-associated abnormalities at the injection sites.

2.2.2.2.4. Other Toxicity Studies

➤ immunogenicity

In the long-term toxicity study involving repeated administration to SD rats for 13 weeks followed by a 4-week recovery period, no anti-AS1501 antibodies were detected in any animals. In the long-term toxicity study involving repeated administration to rhesus monkeys for 13 weeks followed by a 4-week recovery period, anti-drug antibodies were detected in one animal from each of the negative control group, and the test substance dose groups at 30 and 100 mg/kg on days D93, D120, and D-1 (confirmed positive), with titers all <1; one female animal in the negative control group (1725249) exhibited detectable anti-drug antibodies on days D93 and D120 (confirmed positive), with titers of 1:4 and 1:2, respectively. One male animal in the 10 mg/kg test substance dose group (1725253) demonstrated detectable anti-drug antibodies on days D29, D50, and D64 (confirmed positive), with titers of <1, 1:2, and 1:32, respectively. No anti-drug antibodies were detected in the remaining animals during the study. Due to the low antibody titers or the occurrence in the negative control group, these findings are considered to have limited toxicological significance. Furthermore, AS1501 is a human-derived protein and an exogenous protein in rhesus monkeys; therefore, its results cannot be generalized to human use.

➤ **immunotoxicity**

Immunotoxicity in rats

Based on the long-term toxicity study involving 13 weeks of repeated administration and a 4-week recovery period in SD rats, no abnormal changes related to the test substance were observed in the blood cell counts of all animals in Groups 1–4. Compared with the corresponding negative control group, there were no statistically significant differences in white blood cells, neutrophils, or lymphocytes. No statistically significant changes were also noted in the weights of immune system organs such as the thymus, spleen, and lymph nodes. Histopathological examinations of SD rats euthanized at the end of administration and at the conclusion of the recovery period revealed no pathological alterations associated with the test substance. Furthermore, immune cell parameters—including lymphocyte subsets, cytokines, and serum complement levels—showed no abnormalities linked to the test substance.

During the trial, one female animal in the toxicology study's negative control group (Animal No.: 17-25377) died on day D64. Prior to death, it exhibited mild hunchback, pallor of the left ear and limbs, and grossly enlarged liver and spleen. Histopathological examination revealed bone marrow-derived myeloid leukemia with metastases in the liver, spleen, and lungs. Therefore, the cause of death was determined to be multi-organ failure.

No pathological changes associated with the test substance were observed in SD rats undergoing euthanasia at the end of drug administration or at the conclusion of the recovery period. In Group 4, animals 17 – 25465 exhibited renal lesions (renal cell carcinoma) upon gross autopsy following euthanasia at the end of drug administration; animal 17 – 25480 showed renal lesions (renal cell carcinoma); and animal 17 – 25479 displayed a breast mass (adenocarcinoma) during the same procedure.

Given that the negative control group in the study developed myeloid leukemia, rats exhibited a high incidence of tumors during the prolonged toxic period (13 weeks of repeated administration followed by a 4-week recovery phase), and this phenomenon was not observed in the long-term toxicity studies with rhesus monkeys, it is concluded that the aforementioned pathological changes represent spontaneous tumorigenic lesions in SD rats and are unrelated to the test substance.

Immunotoxicity in crab-eating macaques

Based on the long-term toxicity study involving 13 weeks of repeated administration and a 4-week recovery period in cynomolgus monkeys, no abnormal changes related to the test substance were observed in the blood cell counts of all animals. Compared with the corresponding negative control group, there were no statistically significant differences in white blood cells, neutrophils, or lymphocytes. No statistically significant alterations were noted in the weights of immune system organs such as the thymus, spleen, or lymph nodes. Histopathological examinations of SD rats euthanized at the end of administration and at the conclusion of the recovery period also revealed no pathological changes associated with the test substance. Immunological parameters in cynomolgus monkeys—including lymphocyte subsets, cytokines, and serum complement—showed no abnormalities related to the test substance. Gross anatomical examinations at day 93 (post-administration) and day 120 (4 weeks after discontinuation) revealed no abnormalities related to the test substance; histopathological analyses in all animals demonstrated no such abnormalities, and no tumor development was observed.

➤ ADCC Effect Evaluation Test for AS1501

In the ADCC efficacy evaluation study of AS1501, PBMCs were used as effector cells and TRAIL-transfected 293T cells as target cells, with the calcein assay employed to detect the ADCC activity of AS1501. The same batch of PBMCs was utilized throughout the experiment, with Rituxan (a human-mouse chimeric antibody targeting CD20) and Daudi target cells serving as positive system controls,

while an anti-HA human antibody (IgG1) acted as the negative isotype control. Results demonstrated that Rituxan exhibited potent ADCC activity, showing a gradient relationship across different effector-target ratios, whereas AS1501 exhibited no or only weak ADCC activity. In the CD efficacy evaluation study, varying concentrations of AS1501 were added to TRAIL-transfected 293T cells, and the differences in killing efficiency were compared under the presence of 10% normal human serum complement. Given that Rituxan (the human-mouse chimeric antibody targeting CD20) exhibits significant CDC activity, Daudi cells and Rituxan were employed as positive system controls. The results confirmed that Rituxan markedly mediated CDC activity, achieving a specific killing efficiency of up to 100% against Daudi cells, whereas AS1501 showed negligible or no specific killing efficiency.

AS1501 contains the IgG1 Fc region and should theoretically exhibit ADCC and CDC effects. However, in several conducted experiments, these effects were either absent or very weak. This may be attributed to the fact that TRAIL is a death-inducing protein whose receptor is expressed on nearly all cells; high TRAIL expression directly triggers apoptosis, resulting in extremely low surface expression levels that are insufficient to induce ADCC or CDC. Thus, ADCC and CDC likely do not constitute the primary mechanisms through which AS1501 exerts its effects.

2.2.2.3. Pharmacokinetic Studies

2.2.2.3.1. absorb

Pharmacokinetics of a single intravenous infusion in SD rats

This study investigated the pharmacokinetic characteristics of AS1501 following a single intravenous injection in SD rats.

The study involved 36 SD rats (equal numbers of males and females), randomly assigned to low-, medium-, or high-dose groups with six animals per group. Intravenous injections of AS1501 were administered at doses of 4.2 mg/kg, 20 mg/kg, and 42 mg/kg, respectively. Whole blood was collected before administration and at 5 minutes, 2 hours, 8 hours, 24 hours post-administration, as well as on days 2, 3, 5, 7, 11,14,21, and 28, followed by plasma separation. Plasma drug concentrations in each sample were determined using the ELISA method with a detection limit of 2.5 ng/mL. Pharmacokinetic parameters were calculated for each animal using the WinNonlin non-ventricular model (NCA).

During the trial period, no adverse clinical manifestations related to drug administration were observed.

The statistical parameters of plasma pharmacokinetic profiles for each animal group are presented as follows:

Table 1 Pharmacokinetic parameters of AS1501 following a single intravenous infusion in SD rats

group		t1/2	Cmax	AUClast	AUCinf	V	Cl	MRT	AUC (0-168h)
mg/kg		h	µg/mL	h·mg/mL	h·mg/mL	mL/kg	mL/h/kg	h	h·mg/mL
Low dosage Quantity Group 4.2	Mean	100.55	84.19	4.86	4.97	1520.06	0.85	145.23	4.71
	SD	53.29	8.02	0.41	0.46	59.89	0.08	21.19	0.36
Middle dose Quantity Group 20	Mean	166.94	385.74	23.63	24.86	192.67	0.81	159.12	22.46
	SD	34.86	29.14	1.55	1.91	35.64	0.07	9.32	1.30
High dosage Quantity Group 42	Mean	141.66	767.18	46.88	48.80	174.79	0.87	158.85	44.56
	SD	35.39	57.01	3.93	4.63	39.48	0.08	6.01	3.32

When administered intravenously to SD rats at different doses, no significant differences were observed in any pharmacokinetic parameters between genders (p>0.05).

The peak plasma drug concentrations and drug exposure levels in SD rats increased with rising dosages. The dose ratios among low-, medium-, and high-dose groups were 1:4.76:10, respectively, with the ratios of mean plasma Cmax and mean AUC (0–504 h) being 1:4.58:9.11 and 1:4.77:9.46, indicating linear pharmacokinetic characteristics. The average concentration-time curves of the drug in SD rat plasma are shown in the figure below.

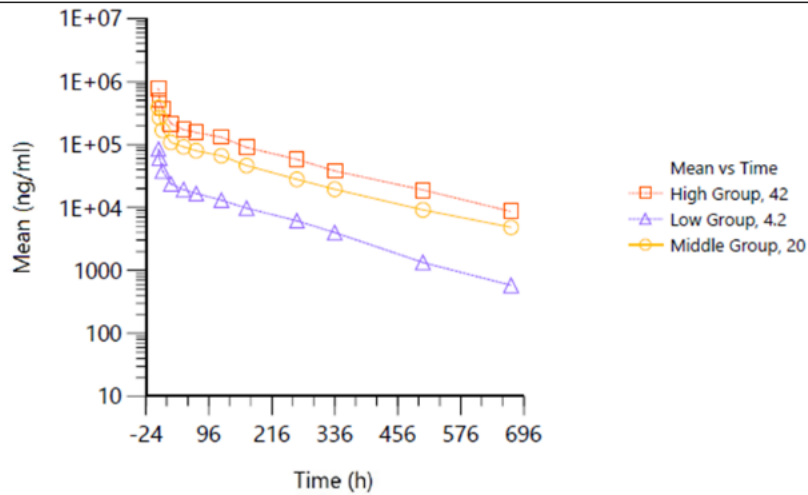


Figure 5 Average drug concentration–time curve in plasma of SD rats

Pharmacokinetics of intravenous administration in cynomolgus monkeys

To investigate the pharmacokinetic characteristics of AS1501 administered via intravenous infusion, preliminary and formal trials were conducted successively in cynomolgus monkeys.

In a formal pharmacokinetic study of AS1501 administered via intravenous infusion in cynomolgus monkeys, 24 monkeys were enrolled (equal numbers of males and females), randomly divided into four groups by sex: low-dose, medium-dose, high-dose groups, and a continuous administration group. The animals received intravenous doses of AS1501 at 3 mg/kg, 10 mg/kg, 30 mg/kg, and 3 mg/kg, respectively (the continuous administration group received the dose once weekly for four consecutive administrations). Blood samples were collected at the following time points for Groups 1–3: before administration, immediately after administration (± 1 min), at 2 and 8 hours post-administration, and on days 1, 2, 3, 5, 7, 10,14,17,21, and 28; for Group 4: before the first administration, immediately after administration (± 1 min), at 2 and 8 hours post-administration, and on days 1, 2, 3, 5, and 7; before the second and third administrations and at 2 hours post-administration; before the fourth (final) administration, immediately after administration (± 1 min), at 2 and 8 hours post-administration, and on days 1, 2, 3, 5, 7, 10,14,17,21, and 28. Plasma was collected at each time point.

No abnormal clinical manifestations were observed in any animals during the trial period. The statistical data of pharmacokinetic parameters of the drug in plasma from all groups are presented in the table below.

Table 1 Pharmacokinetic parameters of AS1501 after single and multiple intravenous administrations in cynomolgus monkeys										
group		t1/2	Cmax	AUClast	AUCinf	V	Cl	MRT	AUC (0-168h)	AF
mg/kg		h	µg/mL	h·mg/mL	h·mg/mL	mL/kg	mL/h/kg	h	h·mg/mL	
Low dosage Quantity Group 3	Mean	103.45	68.62	3.05	3.05	150.97	1.02	51.70	2.89	
	SD	14.96	8.10	0.60	0.60	33.62	0.20	10.42	0.48	
Middle dose Quantity Group 10	Mean	46.44	200.51	12.37	12.37	56.34	0.87	78.52	10.43	
	SD	7.28	31.27	3.40	3.41	11.05	0.29	14.94	2.33	
High dosage Quantity Group 30	Mean	47.25	638.72	45.35	45.39	45.30	0.67	114.65	32.87	
	SD	9.79	91.94	6.95	7.00	7.75	0.10	24.33	2.57	
Continuous Group for the first time 3	Mean	34.11	60.88	2.49	2.58	57.80	1.20	42.86	60.88	
	SD	5.92	10.28	0.42	0.46	8.73	0.23	4.90	10.28	
Continuous Group Last time 3	Mean	87.34	72.14	3.09	3.09	117.68	1.01	48.10	2.97	1.21
	SD	47.90	6.15	0.68	0.68	49.93	0.22	12.41	0.59	0.24

The dosage ratios of the low-dose, medium-dose, and high-dose groups for the test substance were 1:3.33:10, respectively, with the ratios of mean plasma Cmax and mean AUC (0–168 h) being 1:2.92:9.31 and 1:3.60:11.35, respectively. Post-administration regression analyses of Cmax versus dose and AUC (0–168 h) versus dose yielded slopes of 0.97 and 1.06, with 90% confidence intervals of 1.05–

0.89 and 1.15–0.97, respectively; R^2 values were 0.98 and 0.97, indicating linear pharmacokinetic characteristics following a single intravenous injection of the test substance in cynomolgus monkeys within the dose range of 3–30 mg/kg. The average plasma concentration-time curve of the test substance is shown in the figure below.

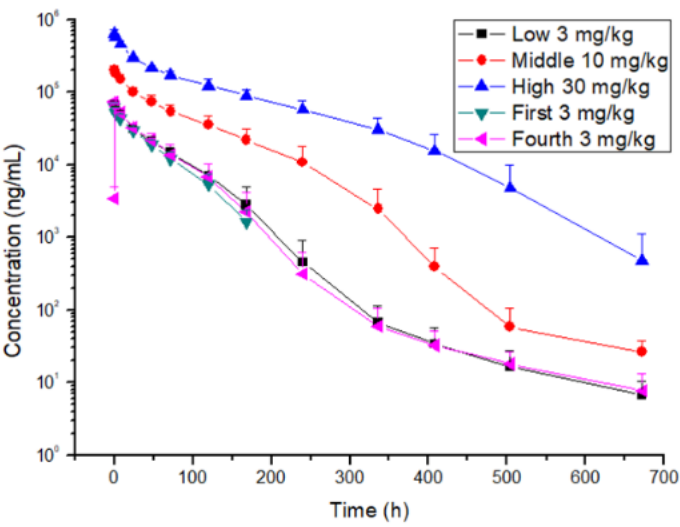


Figure 5 Average plasma drug concentration-time curve for the test sample

The test substance was administered via intravenous injection to cynomolgus monkeys once weekly (3 mg/kg) for four consecutive doses, yielding an average accumulation factor (AUC(0–168 h) after four doses/AUC(0–168 h) after the first dose) of 1.21.

2.2.2.3.2. Distribution and Excretion

In a distribution and excretion study of radionuclide ^{125}I -labeled AS1501 administered intravenously to SD rats, the method of TCA protein precipitation combined with SHPLC was employed to investigate the distribution of the isotope (^{125}I)-labeled AS1501 in postnatal tissues as well as its excretion in feces, urine, and bile.

The study randomized 36 SD rats into 6 groups of 6 each, with equal numbers of males and females. The groups included: 4 tissue distribution groups (time points: 10 min, 2 h, 24 h, and 168 h; dose: 4.2 mg/kg) and 2 excretion groups (bile, feces, and urine; dose: 4.2 mg/kg).

result display :

(1) Following intravenous administration of 125I-AS1501, the tissues ranked by total radioactivity AUC from highest to lowest are (see Figure 11): plasma, thyroid, spleen, lung, kidney, gonads, heart, liver, stomach, pancreas, small intestine, muscle, fat, and brain; ranked by precipitation radioactivity AUC from highest to lowest are: plasma, thyroid, spleen, lung, kidney, gonads, heart, liver, pancreas, small intestine, stomach, fat, muscle, and brain (urinary AUC data unavailable). Following administration, 125I-AS1501 is primarily distributed in plasma, followed by the spleen, lung, kidney, gonads, heart, liver, pancreas, small intestine, stomach, fat, and muscle, with minimal distribution in the brain.

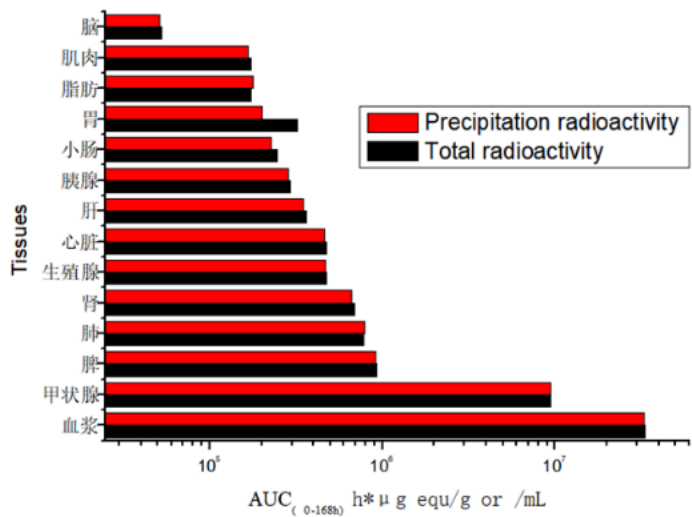


Figure 6 Distribution 5of the drug in tissues and plasma

- (2) After intravenous administration of 125I-AS1501 to rats, the radiopharmaceutical was primarily excreted in urine, with small amounts excreted via feces and minimal excretion through bile. At 17 days post-injection, urinary and fecal excretion accounted for 93.65 ± 4.94% and 3.28 ± 0.74% of the injected radioactivity, respectively; combined urinary and fecal excretion represented 96.94 ± 5.37% of the total injected radioactivity. Within 8 hours after intravenous administration, bile excretion accounted for 0.57 ± 0.22% of the injected radioactivity.
- (3) The plasma concentration of 125I-AS1501 (precipitated radioactivity) peaks at 10 minutes post-administration and declines over time. In other tissues, precipitated radioactivity remains stable at a plateau level for approximately 24 hours after administration and gradually decreases

thereafter until 168 hours. In cardiac tissue, the level rises slightly at 2 hours post-administration compared to 10 minutes post-administration.

Radioactivity levels gradually increase over time following intrathyroid administration, reaching a peak at 168 hours due to thyroid iodine uptake; therefore, this does not reflect the actual distribution of 125I-AS1501 within the tissue.

2.2.2.3.3. Other Pharmacokinetics

Toxicokinetics of AS1501 administered via repeated intravenous infusion in SD rats

This study employed 72 SD rats, with equal numbers of males and females, divided into a negative control group and low-, medium-, and high-dose groups of the test substance. The animals received intravenous infusions of either the negative control substance or the test substance at doses of 20 mg/kg, 60 mg/kg, and 200 mg/kg, respectively. Administration was performed once weekly via intravenous infusion for a total of 13 weeks. Blood samples were collected from the negative control group on days D1 and D85 before administration and 2 hours post-administration to isolate plasma. For the low-, medium-, and high-dose groups, blood samples were collected on days D1 and D85 before administration, at 3 minutes (± 1 minute) after administration, and at 2, 8, 24, 48, 72, 120, and 168 hours after administration to isolate plasma.

The results demonstrated that the temporal trends of plasma drug concentrations were largely consistent across animals of different genders in all groups, with plasma drug concentrations showing a positive correlation with administered doses in low-, medium-, and high-dose groups. Pharmacokinetic parameters were calculated using the WinNonLin 6.4 non-ventricular model (NCA). The pharmacokinetic parameter results for each group are presented as follows (see Table 14).

In animals administered low, medium, and high doses of the test substance (20, 60, and 200 mg/kg), both C_{max} and AUC_{last} showed positive correlations with the dosing dose. After weekly administration for 13 consecutive doses, the plasma drug accumulation factors (AUC_{last} at 13th dose vs. AUC_{last} at first dose) were 3.18, 3.78, and 2.55 in male animals across all dose groups, respectively; while those in female animals were 2.73, 2.15, and 1.77.

Table 1 Toxicokinetic parameters of repeated intravenous infusion of AS1501 in SD rats over 13 weeks

Period	Group	Sex	Tmax	Cmax	SE_Cmax	AUClast	SE_AUClast	AUCinf
	mg/kg		h	mg/mL	mg/mL	h·mg/mL	h·mg/mL	h·mg/mL
D1	Low-dose group	M	0.05	0.42	0.01	15.76	0.33	24.23
		F	0.05	0.4	0.02	14.82	0.41	24.35
	Medium-dose group	M	0.05	1.08	0.02	37.44	0.92	53.22
		F	0.05	1.07	0.04	44.82	0.82	61.22
	High-dose group	M	0.05	3.64	0.09	129.86	2.82	209.42
		F	0.05	3.65	0.25	142.77	1.08	235.77
D85	6	M	0.05	0.77	0.04	50.09	2.42	98.6
		F	0.05	0.63	0.03	40.39	1.04	82.4
	7	M	0.05	1.98	0.08	141.59	3.78	293.11
		F	0.05	1.74	0.08	96.15	5.07	182.08
	8	M	0.05	6.25	0.25	331.68	10.27	671.68
		F	0.05	5.43	0.24	252.61	11.56	492.18

Toxicokinetics of AS1501 administered via repeated intravenous infusion in cynomolgus monkeys

The study involved 40 crab-eating macaques, randomly divided into four groups with five males and five females in each group. The negative control group received sodium chloride solution; the low-, medium-, and high-dose test group received repeated intravenous infusions of the test substance at doses of 10 mg/kg, 30 mg/kg, and 100 mg/kg, respectively. Administration was performed once weekly for a total of 13 weeks.

Table 1 Toxicokinetic parameters of AS1501 following repeated intravenous infusion in crab-eating macaques over 13 weeks

period	group		Cmax	AUClast	AF
	Dosage (mg/kg)		mg/mL	h·mg/mL	
First administration	Low-dose group (n=10)	Mean	0.21	11.38	
	10	SD	0.04	1.12	
	Middle-dose group (n=10)	Mean	0.72	37.28	
	30	SD	0.07	5.10	

period	group		Cmax	AUClast	AF
	Dosage (mg/kg)		mg/mL	h·mg/mL	
13 times dose	High-dose group (n=10) 100	Mean	2.15	122.91	
		SD	0.41	12.17	
	Low-dose group (n=10) 10	Mean	0.35	23.03	2.11
		SD	0.06	6.85	0.14
	Middle-dose group (n=10) 30	Mean	1.12	77.37	2.11
		SD	0.14	12.50	0.23
	High-dose group (n=10) 100	Mean	3.12	210.94	1.71
		SD	0.39	38.63	0.24

Blood samples of approximately 1 mL were collected from the subcutaneous veins of the hind limbs in animals D1 and D85, followed by plasma separation. In the negative control group, blood samples were collected at pre-administration time and at 2 hours post-administration initiation. In the test group, samples were collected before administration, immediately after administration completion, as well as at 0.5 h, 2 h, 8 h, 24 h, 48 h, 72 h, 120 h, and 168 h post-administration. The toxicokinetic parameters of AS1501 were calculated using the WinnonLin 6.4 non-ventricular model (NCA) (see Table 10).

In the low-, medium-, and high-dose groups (10,30, and 100 mg/kg) of test subjects, the dose ratios were 1:3:10. Following the initial administration, the ratios of mean plasma drug Cmax and AUClast among these groups were 1:3.35:10.01 and 1:3.28:10.80, respectively; after 13 administrations, the corresponding ratios were 1:3.2:8.91 and 1:3.36:9.16. These results demonstrate that both plasma peak drug concentrations and plasma drug exposure are positively correlated with the administered doses.

Animals in the low-, medium-, and high-dose groups of the test substance were administered once weekly for four consecutive weeks, resulting in accumulation factors (AUClast, last dose/AUClast, first dose) of 2.11,2.11, and 1.71, respectively.

2.2.2.4. Other in vitro tests
2.2.2.4.1. In vitro receptor occupancy assay

The test procedure is as follows:

(1) PBMC preparation: Take a lyophilized PBMC sample (source: whole blood from healthy volunteers), rapidly thaw it in a 37°C water bath, transfer the cell suspension to a 15 ml centrifuge tube, add 2 ml of complete culture medium (RPMI-1640 + 10% FBS), gently vortex to mix, and centrifuge at

1500 rpm at room temperature for 5 minutes; then resuspend the cells in RPMI-1640, count them, and adjust the cell density to $10 \times 10^6/\text{mL}$.

(2) Prepare 12 clean 5 ml flow cytometry tubes and number them as follows: tubes 1–10 contain AS1501 test samples at concentrations of 1000 g/mL, 500 g/mL, 200 g/mL, 100 g/mL, 50 g/mL, 25 g/mL, 12.5 g/mL, 6.25 g/mL, 3.125 g/mL, and 1.56 g/mL respectively; tube 11 serves as the negative control with physiological saline; tube 12 is the homologous antibody control. Add the cell suspension, AS1501 test sample, physiological saline, and RPMI-1640 culture medium in the order specified in the table below, mix gently by pipetting, and incubate at room temperature for 30 minutes.

Group Number Excipients formulations or	Test Sample A		Test Sample B				Test Sample C				negative control group	Test Sample B
	1	2	3	4	5	6	7	8	9	10	11	12
cell suspension (1)	50	50	50	50	50	50	50	50	50	50	50	50
AS1501 Test Sample ()	10	5	20	10	5	2.5	10	5	2.5	1.25	-	20
normal saline (1)	10	15	-	10	15	17.5	10	15	17.5	18.75	20	-
RPMI-1640	30	30	30	30	30	30	30	30	30	30	30	30

(3) Upon completion of incubation, centrifuge the sample at 1500 rpm at room temperature for 5 minutes to collect cells. Wash the cells twice with FACS buffer (PBS + 1% BSA + 0.5 mM EDTA), then add 100 L each of the following solutions to tubes 1–11: FACS buffer diluted with 2% Rat Serum containing Alexa Fluor 647 anti-human IgG Fc antibody (1:200 dilution); and tube 12 receives an equal volume of Alexa Fluor 647 Mouse IgG1 isotype control antibody. Mix gently and incubate at room temperature for 30 minutes.

(4) After completing the flow cytometry antibody staining, wash the cells twice with FACS buffer, centrifuge at 1500 rpm for 5 minutes, and discard the supernatant. Add 100 L of diluted PI dye (97 L PBS + 3 L PI) to each tube and incubate at room temperature for 15 minutes to remove dead

cells. Following incubation, resuspend the cells in 100 L of FACS buffer and immediately analyze the levels of TRAIL bound by AS1501 on live cells (as expressed as the proportion of hIgG Fc+ cells among live cells) and the total TRAIL level on cell surfaces (as expressed as the proportion of hIgG Fc+ cells among live cells at saturation concentration) using a flow cytometer to calculate receptor occupancy.

Receptor occupancy calculation formula:

$$\text{Receptor occupancy (\%)} = (\text{hIgG Fc} + \text{t-hIgG Fc} + \text{nc}) / (\text{hIgG Fc} + \text{o-hIgG Fc} + \text{nc}) \times 100\%$$

hIgG Fc+ ratio: percentage of hIgG Fc+ cells among live cells in different concentration groups of AS1501 test samples;

hIgG Fc+ nc: Ratio of hIgG Fc+ cells to live cells in the saline control group;

hIgG Fc+ ratio: the proportion of hIgG Fc+ cells among live cells in the saturated concentration group.

Test results: Binding of AS1501 samples at different concentrations to PBMCs

In this experiment, compared to the saline control group, the proportion of PBMCs bound to AS1501 increased progressively with rising AS1501 concentration during incubation; however, this increase ceased when the concentration reached or exceeded 200 g/ml (Figure 13). This indicates that AS1501 binding to TRAIL on cell surfaces had reached saturation, meaning all TRAIL molecules on immune cells were fully neutralized by AS1501.

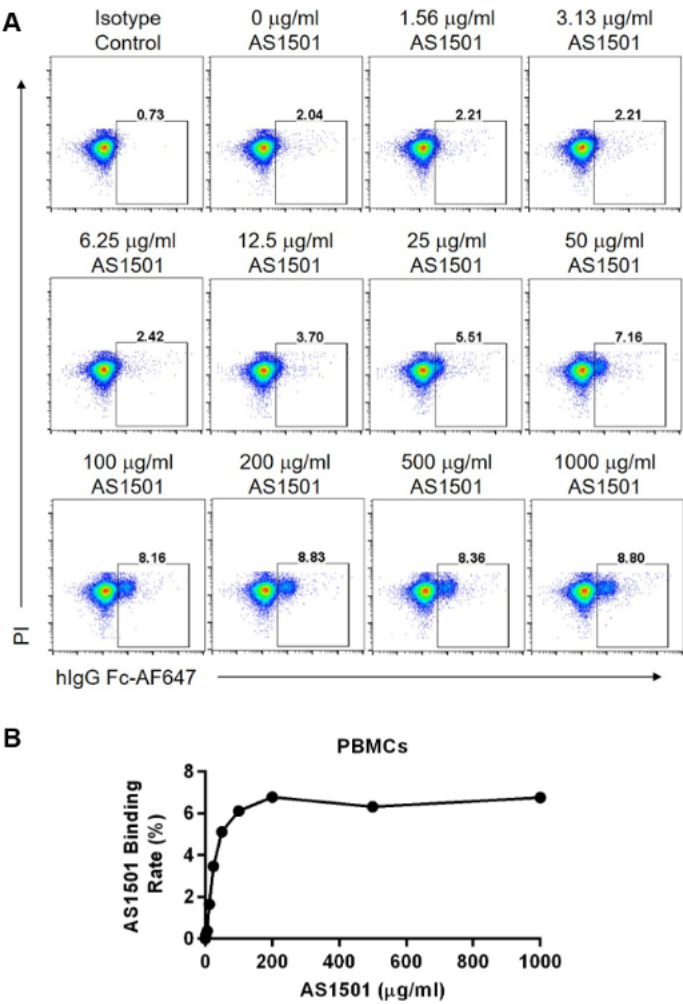


Figure 6 Comparison of binding efficiency between AS1501 at different concentrations and PBMCs
(A) Representative flow cytometry plot; (B) Statistical analysis plot.

Test results: Occupation of TRAIL on PBMC surfaces by test samples of AS1501 at different concentrations

The receptor levels measured at a saturated concentration of 200 $\mu\text{g/ml}$ AS1501 were used as the total TRAIL levels on PBMC surfaces. Using the formula, the receptor occupancy rates for AS1501 samples ranging from 6.25 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$ are shown in Figure 14.

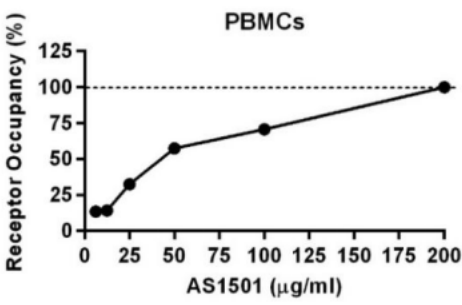


Figure 7: Occupation 6of TRAIL on PBMC surfaces by AS1501 at different concentrations

2.2.2.4.2. In vitro stimulation of human PBMCs to release cytokines (IL-2, IFN-γ)

The test procedure is as follows:

(1) PBMC preparation: Take two aliquots of cryopreserved PBMCs (source: whole blood from healthy volunteers), rapidly thaw them in a 37°C water bath, transfer the cell suspension to a 15 mL centrifuge tube, add 13 mL of RPMI-1640 culture medium, gently vortex to mix, and centrifuge at 1500 rpm at room temperature for 10 minutes; discard the supernatant, wash the cells once with 14 mL of RPMI-1640 medium, resuspend the cells in RPMI-1640, count the cells, and adjust the cell density to 5 × 10⁶/mL.

(2) After thoroughly mixing the PBMCs, distribute them into a 96-well flat-bottom plate with 1 × 10⁶ cells per well (i.e., 200 μL of cell suspension). Allow the PBMCs to rest in a 37°C incubator for 1 hour, then stimulate them with concentrations of AS1501 ranging from 0 g/mL to 240 g/mL, along with 0.5 g/mL anti-human CD3e antibody, for 0.5 h, 6 h, or 24 h.

group or Excipients formulations	0μg/ml	15μg/ml	30 μg/ml	60 μg/ml	120μg/ml	240μg/ml	0.5 μ g/ml anti- human CD3e
cell suspension (μ l)	200	200	200	200	200	200	200
AS1501 Test Sample A (μl)	0	0.3	0.60	1.21	2.43	4.92	0

normal saline (μl)	4.92	0	0	0	0	0	0
Anti-human CD3e (μl)	0	0	0	0	0	0	0.1

(3) Upon completion of incubation, centrifuge at 400 g for 5 minutes at room temperature, collect the supernatant in a clean centrifuge tube, and store frozen at -80°C.

(4) Subsequently, the concentrations of IL-2 and IFN-γ in the culture supernatant were measured.

Experimental results: Treatment of PBMCs with AS1501 at different concentrations did not induce significant release of IL-2 or IFN-γ.

After incubation of human PBMCs with AS1501 at different concentrations for 0.5 h, 6 h, or 24 h in vitro, no IL-2 was detected in the supernatants. As a positive control, only after 24-hour treatment did a high concentration of IL-2 (109 pg/mL) become detectable in the supernatant of the group treated with 0.5 μg/mL anti-human CD3e (Figure 15). Similarly, after 0.5 h or 6 h of in vitro stimulation with AS1501 at various concentrations, the IFN-γ levels in the culture supernatants were extremely low (<20 pg/mL). In the AS1501-treated group at 24 h, significant IFN-γ levels (>20 pg/mL) were observed only at high concentrations (120 μg/mL and 240 μg/mL). As a positive control, supernatant IFN-γ levels were minimal after 0.5 h or 6 h treatment with 0.5 μg/mL anti-human CD3e; however, after 24 h treatment, the supernatant IFN-γ concentration reached 438 pg/mL. In conclusion, AS1501 treatment of PBMCs does not induce significant release of IL-2 or IFN-γ.

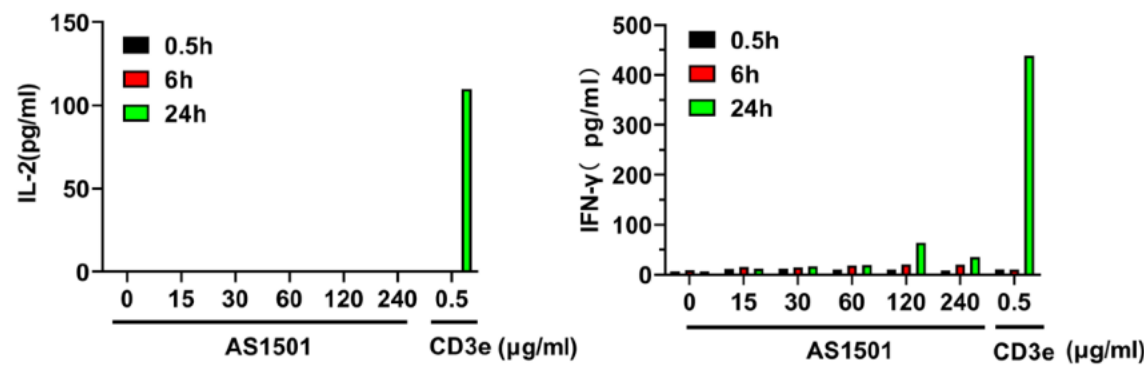


Figure 7 Detection of IL-2 and IFN-γ release from human peripheral blood mononuclear cells induced by AS1501 treatment

The ELISA assay determined the concentrations of IL-2 and IFN-γ released into the supernatant by human PBMCs after treatment with AS1501 in vitro. A positive control group was treated with 0.5 μg/ml anti-human CD3e for the same duration.

1.1.1.1.1. In vitro stimulation assay for detecting cytokine (IL-6) release from human PBMCs

The test procedure is as follows:

- (1) Preparation of PBMCs: Take a lyophilized sample of PBMCs (source: whole blood from healthy volunteers), rapidly thaw it in a 37 °C water bath, transfer the cell suspension to a 15 ml centrifuge tube, add 10 ml of RPMI-1640 culture medium, gently vortex to mix, and centrifuge at 1500 rpm at room temperature for 10 minutes; then discard the supernatant, wash the cells once with 10 ml of RPMI-1640 culture medium, resuspend the cells in RPMI-1640, count them, and adjust the cell density to 10×10⁶/mL.
- (2) After thoroughly mixing the PBMCs, plate them onto a 96-well U-shaped plate with 1 × 10⁶ cells per well (i.e., 100 μl of cell suspension). Allow the PBMCs to rest in a 37°C incubator for 1 hour, then stimulate them with either 0 g/mL, 30 g/mL, 60 g/mL, 120 g/mL, or 240 g/mL of AS1501, or 0.5 g/mL of anti-human CD3e antibody for 0.5 h, 6 h, or 24 h respectively.

group Excipients formulations or	0μg/ml	30μg/ml	60 μg/ml	120 μg/ml	240μg/ml	0.5 μ g/ml anti-human CD3e
cell suspension (μl)	100	100	100	100	100	100
AS1501 Test Sample A (μl)	0	0.30	0.60	1.21	2.46	0
normal saline (μl)	2.46	0	0	0	0	0
Anti-human CD3e (μl)	0	0	0	0	0	0.05

(3) Upon completion of incubation, centrifuge at 400 g for 5 minutes at room temperature, collect the supernatant in a clean centrifuge tube, and store frozen at-80°C.

(4) Subsequently, the IL-6 concentration in the culture supernatant was measured.

Experimental results: Treatment of PBMCs with AS1501 induced significant release of IL-6.

As shown in Figure 16, human PBMCs did not release IL-6 when treated with AS1501 at different concentrations for 0.5 hours. However, after 6-hour treatment with 30 μg/ml AS1501, IL-6 release began; the release increased rapidly with 60 μg/ml AS1501, and reached its peak at 120 μg/ml. After 24-hour treatment, significant amounts of IL-6 were also detected in the supernatant. Notably, 30 μg/ml AS1501 stimulated the release of up to 317 pg/mL IL-6 after 24 hours, while higher concentrations (>200 pg/mL) similarly induced substantial IL-6 release. As a positive control, 0.5 μg/ml anti-human CD3e treatment for 6 hours resulted in approximately 100 pg/mL IL-6 release from PBMCs, with supernatant levels reaching about 550 pg/mL after 24 hours. In conclusion, the results demonstrate that AS1501 binds to receptors on PBMCs, triggering the release of the pro-inflammatory cytokine IL-6, and this effect is observed even at low concentrations (30 μg/ml).

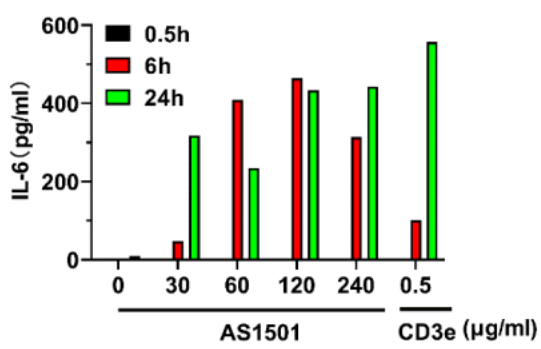


Figure 7 Detection of IL-6 release from human peripheral blood mononuclear cells induced by AS1501 treatment

The ELISA assay measured the concentration of IL-6 released into the supernatant by human PBMCs after treatment with AS1501 in vitro, using 0.5 µg/mL anti-human CD3e treated for the same duration as a positive control.

1.1.1.1.1. Test for detecting the release of cytokines (TNF-α) and apoptosis in human PBMCs under in vitro stimulation

The test procedure is as follows:

(1) PBMC preparation: Take a lyophilized PBMC sample (source: whole blood from healthy volunteers), rapidly thaw it in a 37°C water bath, transfer the cell suspension to a 15 mL centrifuge tube, add 10 mL of RPMI-1640 culture medium, gently vortex to mix, and centrifuge at 1500 rpm at room temperature for 10 minutes; discard the supernatant, wash the cells once with 10 mL of RPMI-1640 culture medium, resuspend the cells in RPMI-1640, count them, and adjust the cell density to 10 × 10⁶/mL.

(2) After thoroughly mixing the PBMCs, plate them onto a 96-well U-shaped plate with 1 × 10⁶ cells (100 µl of cell suspension per well). Allow the PBMCs to rest in a 37°C incubator for 1 hour, then stimulate them with either 0 g/mL, 30 g/mL, 60 g/mL, 120 g/mL, or 240 g/mL of AS1501, or 0.5 g/mL of anti-human CD3e antibody for 0.5 h, 6 h, or 24 h.

group Excipients formulations or	0μg/ml	30μg/ml	60 μg/ml	120 μg/ml	240μg/ml	0.5 μ g/ml anti-human CD3e
cell suspension (μl)	100	100	100	100	100	100
AS1501 Test Sample A (μl)	0	0.30	0.60	1.21	2.46	0
normal saline (μl)	2.46	0	0	0	0	0
Anti-human CD3e (μl)	0	0	0	0	0	0.05

(3) Upon completion of incubation, centrifuge at 400 g for 5 minutes at room temperature; collect the supernatant in a clean centrifuge tube and store frozen at −80°C. Collect the cells and wash them twice with pre-cooled 1 × PBS at 500×g for 5 minutes at 4°C, then discard the supernatant. Resuspend the cells in 100 μl of pre-cooled 1 × Annexin V Binding Buffer; add 5 μl of Annexin V and 5 μl of PI to each sample, mix gently, and incubate at room temperature away from light for 15 minutes. After incubation, add 400 μl of pre-cooled 1 × Annexin V Binding Buffer to each sample, mix gently, place the samples on ice, and perform flow cytometry analysis within 1 hour.

(4) Subsequently, the TNF-α concentration in the culture supernatant was measured.

Test results: Flow cytometry analysis demonstrated that AS1501 does not induce significant apoptosis in PBMCs.

In this experiment, different concentrations of AS1501 stimulated human peripheral blood mononuclear cells (PBMCs) in vitro for 0.5 h, 6 h, or 24 h without inducing significant cell death; in contrast, treatment with 0.5 μ g/ml anti-human CD3e served as a positive control and induced pronounced apoptosis (Figure 17A).

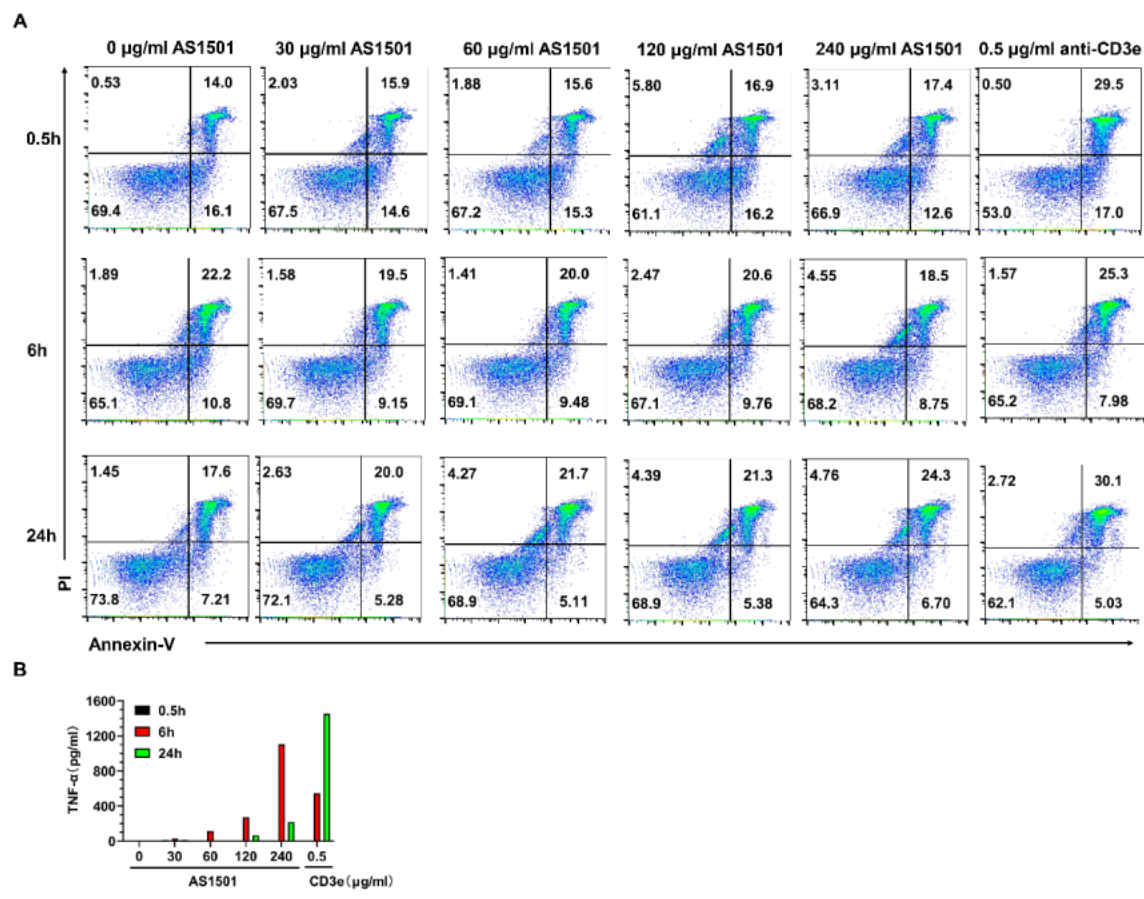


Figure 7 Detection of AS1501-induced apoptosis and TNF-α release in human peripheral blood mononuclear cells

(A) Flow cytometry analysis of apoptosis in human PBMCs treated with AS1501, using anti-human CD3e as the positive control; (B) ELISA assay quantifying TNF-α released into the supernatant from human PBMCs after in vitro treatment with AS1501, with 0.5 μg/mL anti-human CD3e treated for the same duration serving as the positive control.

1.1.1.1.1. In vitro stimulation assay for detecting cytokine (IL-10) release from human PBMCs

The test procedure is as follows:

(1) PBMC preparation: Take a vial of frozen PBMCs, rapidly thaw them in a 37°C water bath, transfer the cell suspension to a 15 mL centrifuge tube, add 10 mL of RPMI-1640 culture medium, gently vortex to mix, and centrifuge at 1500 rpm at room temperature for 10 minutes; discard the supernatant, wash the cells once with 10 mL of RPMI-1640 culture medium, resuspend the cells in RPMI-1640, count the cells, and adjust the cell density to 5×10⁶/mL.

(2) After thoroughly mixing the PBMCs, distribute them into a 96-well flat-bottom plate at a density of 5×10^5 cells per well (i.e., 100 μ l of cell suspension). Culture the PBMCs in a 37°C incubator for 1 hour, then stimulate them with either 0 g/mL, 30 g/mL, 60 g/mL, 120 g/mL, or 240 g/mL of AS1501, or 0.5 g/mL of anti-human CD3e antibody for 0.5 h, 6 h, or 24 h respectively.

group Excipients or formulations	0 μ g/ml	30 μ g/ml	60 μ g/ml	120 μ g/ml	240 μ g/ml	0.5 μ g/ml anti-human CD3e
cell suspension (μ l)	100	100	100	100	100	100
AS1501 Test Sample A (μ l)	0	0.30	0.60	1.21	2.46	0
normal saline (μ l)	2.46	0	0	0	0	0
Anti-human CD3e (μ l)	0	0	0	0	0	0.05

(3) Upon completion of incubation, centrifuge at 400 g for 5 minutes at room temperature, collect the supernatant in a clean centrifuge tube, and store frozen at -80°C.

(4) Determination of IL-10 concentration in the post-culture supernatant.

Experimental results: Treatment of PBMCs with AS1501 did not induce IL-10 release.

As shown in Figure 18, no IL-10 was detected in the supernatant after treatment with AS1501 at different concentrations for 0.5 h, 6 h, or 24 h. As a positive control, treatment with 0.5 μ g/ml anti-human CD3e for 24 h also resulted in minimal IL-10 release from PBMCs, approximately 1.7 pg/mL. In conclusion, the experimental results demonstrate that AS1501 does not induce IL-10 release from PBMCs under in vitro stimulation conditions.

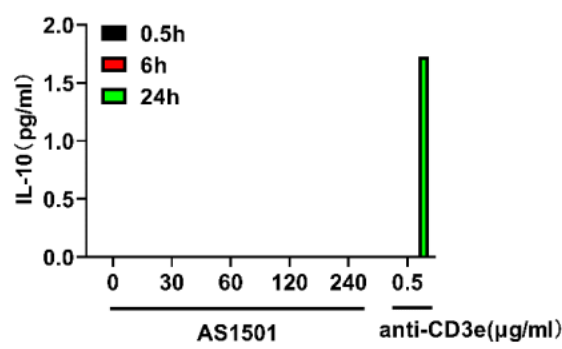


Figure 7. Detection of IL-10 release from human peripheral blood mononuclear cells induced by AS1501 treatment

The ELISA assay measured the concentration of IL-10 released into the supernatant by human PBMCs after in vitro treatment with AS1501, using 0.5 µg/mL anti-human CD3e treated for the same duration as the positive control.

1.1.2. Overview of existing studies on the drug under investigation

A single-center, randomized, double-blind, placebo-controlled, single-dose, dose-escalation study of AS1501 for injection has been conducted in healthy subjects. A total of 314 healthy adult participants were enrolled; 32 were randomly assigned, with 28 receiving the investigational drug and 27 completing the trial as per protocol requirements. Five participants withdrew early (all deemed by investigators unsuitable for continued treatment). The study included eight dose groups: 1 mg/kg, 2 mg/kg, 3 mg/kg, 4.5 mg/kg, 6 mg/kg, 8 mg/kg, 10 mg/kg, and 12 mg/kg. The actual single-dose escalation trial evaluated dose increments from 1 to 3 mg/kg across these groups.

Adverse Events: Among the 28 enrolled subjects receiving the study drug, adverse events (AEs) occurred in 82.14% (23 cases, 76 instances), predominantly grade 1–2; grade ≥ 2 AEs occurred in 28.57% (8 cases, 15 instances), primarily characterized by decreased lymphocyte counts; grade ≥ 3 AEs accounted for 25.00% (7 cases, 8 instances), also involving lymphocytopenia; 3.57% (1 case, 1 instance) of subjects experienced AEs leading to study discontinuation (cytokine release syndrome, likely related to the study drug). Serious adverse events (SAEs) occurred in 7.14% (2 cases, 2 instances) prior to adjusting infusion rates; these were classified as SUSAR and did not recur after rate reduction or dexamethasone pretreatment. No grade ≥ 4 AEs were reported. By dose group, AE incidence was as follows: placebo group: 100% (7 cases, 12 instances); 1 mg/kg: 50% (3 cases, 5 instances); 2 mg/kg: 83.33% (5 cases, 16 instances); 3 mg/kg: 88.89% (8 cases, 43 instances). A preliminary dose-dependent

trend was observed in AE incidence during the single-dose escalation study, with higher AE rates in the placebo group. At least two subjects experienced adverse events (AEs) primarily involving abnormal blood test parameters, predominantly grade 1 in severity, including elevated neutrophil counts (32.14%), decreased lymphocyte counts (21.43%), and elevated interleukins (IL-6, IL-10) (14.29%). Other reported AEs included headache (10.71%), dizziness (7.14%), chest discomfort (7.14%), hypersensitivity reactions (7.14%), cytokine release syndrome (7.14%), and rash (7.14%). Grade 2–3 AEs comprised grade 3 hypersensitivity reactions (2 cases, 2 instances), grade 2 cytokine release syndrome (2 cases, 2 instances), and grade 2 lymphopenia (3 cases, 3 instances); all resolved completely relatively quickly. AEs were summarized according to systemic organ classification and preferred terminology as follows: 1) Common adverse events (occurrence rate $\geq 20\%$) across all dose groups classified by preferred terminology are summarized as follows: 1 mg/kg dose group: all single occurrences with an incidence $<20\%$; 2 mg/kg dose group: decreased lymphocyte count (33.33%), oral mucositis (33.33%), hypersensitivity reactions (33.33%), rash (33.33%); 3 mg/kg dose group: decreased lymphocyte count (77.78%), elevated neutrophil count (77.78%), elevated interleukin levels (44.44%), headache (33.33%), elevated blood triglycerides (22.22%), elevated monocyte count (22.22%), cytokine release syndrome (22.22%); Placebo group: decreased lymphocyte count (28.57%), elevated interleukin levels (28.57%). 2) Summary of common adverse events (incidence $\geq 20\%$) by system/organ classification across all dose groups is as follows: 1 mg/kg dose group: systemic diseases and various reactions at the administration site (33.33%); 2 mg/kg dose group: various laboratory tests (66.67%), gastrointestinal disorders (33.33%), immune system disorders (33.33%), skin and subcutaneous tissue disorders (33.33%); 3 mg/kg dose group: various laboratory tests (88.89%), neurological disorders (44.44%), gastrointestinal disorders (33.33%), immune system disorders (22.22%), respiratory, thoracic, and mediastinal disorders (22.22%); Placebo group: various laboratory tests (71.43%).

Adverse reactions: Among the 28 enrolled subjects who received the study drug, adverse reactions occurred in 60.71% (17 cases, 42 occurrences), predominantly grade 1–2; the incidence of grade ≥ 2 adverse reactions was 17.86% (5 cases, 8 occurrences); and that of grade ≥ 3 adverse reactions was 10.71% (3 cases, 3 occurrences). By group analysis, the incidence rates of adverse reactions were as follows in the placebo group and the 1 mg/kg, 2 mg/kg, and 3 mg/kg dose groups: 57.14% (4 cases, 6 occurrences), 33.33% (2 cases, 3 occurrences), 50.00% (3 cases, 10 occurrences), and 88.89% (8 cases,

23 occurrences), respectively. These results suggest a preliminary dose-dependent trend in adverse reaction incidence with increasing doses in the single-dose escalation study, with a higher incidence observed in the placebo group. At least two subjects experienced adverse reactions primarily involving abnormalities in complete blood count parameters, including elevated neutrophil counts (32.14%), decreased lymphocyte counts (21.43%), and elevated interleukins (IL-6, IL-10) (14.29%). Other adverse reactions included headache (10.71%), dizziness (7.14%), chest discomfort (7.14%), hypersensitivity reactions (7.14%), cytokine release syndrome (7.14%), and rash (7.14%), most of which were grade 1 in severity; grade 2–3 adverse reactions comprised: 2 cases with grade 3 hypersensitivity reactions, 2 cases with grade 2 cytokine release syndrome, and 3 cases with grade 2 decreased lymphocyte counts, all of which resolved completely relatively quickly.

Specific adverse events of particular concern: 1) Hypersensitivity reactions: During the early phase of this trial, the total infusion duration was 30 minutes. Two cases of severe hypersensitivity reactions occurred in the 2 mg/kg dose group, classified as Grade 3 according to CTCAE 5.0 criteria, which investigators determined were most likely related to the investigational drug; both subjects recovered completely after pharmacological and non-pharmacological treatment. In subsequent trials, the total infusion duration was extended to 2 hours, and some participants in the 3 mg/kg dose group received pre-treatment with dexamethasone (5–10 mg) and antihistamines (oral diphenhydramine), resulting in no further hypersensitivity reactions or serious adverse events. 2) Cytokine release syndrome: Two cases of cytokine release syndrome were reported in the 3 mg/kg dose group, graded as Level 2 per CTCAE 5.0 criteria, also considered highly likely drug-related; one participant (without pre-treatment) discontinued treatment but recovered completely after therapy, while another participant (with pre-treatment) completed intravenous administration of the investigational drug and achieved spontaneous recovery.

In the first three dose groups, blood concentrations of TRAIL (the protein targeted by this drug) exhibited a significant inhibitory trend following administration, with clearance rates reaching 100% in groups 2 and 3. A clear trend of increasing drug exposure was observed as the dose increased (from 1 to 3 mg/kg). Following discussions between the sponsor and investigators, combined with safety data from the trial, it was concluded that single-dose administration at doses ranging from 1 mg/kg to 3 mg/kg demonstrated favorable safety and tolerability; consequently, escalation trials for subsequent dose groups (>3 mg/kg) were discontinued.

1.1.3. Overview of Research on Similar Drugs

There remains a significant unmet clinical need for the treatment of relapsed/refractory advanced solid tumors and lymphomas. Cancer therapeutic strategies aim to address two key characteristics of cancer: excessive proliferation and anti-apoptosis. Inducing apoptosis has the potential to eradicate cancer cells, thereby offering opportunities for cancer cure. The TRAIL-DR pathway plays a critical role in the endogenous apoptosis pathway; consequently, drug development targeting this pathway has primarily focused on relapsed/refractory advanced solid tumors and lymphomas with resistance to apoptosis induction. Currently, no specific inhibitor antibodies targeting TRAIL-DR5 that inhibit the apoptosis pathway are available. Three classes of targeted agents are currently under clinical investigation: recombinant forms of TRAIL (e.g., Dulanermin), agonistic antibodies specifically targeting TRAIL-DR4 (mapatumumab), and agonistic antibodies specifically targeting TRAIL-DR5 (Conatumumab [AMG-655]). Multiple clinical trials have been conducted for these agents in relapsed/refractory advanced solid tumors and lymphomas, with favorable overall safety profiles reported [41].

1.2. Risk/Benefit Assessment

AS1501 is a "recombinant human soluble death receptor 5 antibody fusion protein," abbreviated as sDR5-Fc. The sDR5 domain of AS1501 binds to its ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). However, due to the absence of a transmembrane region and an intracellular death domain, sDR5 cannot transmit apoptotic signals intracellularly. Consequently, sDR5 competes with death receptors on the cell membrane for TRAIL binding, thereby inhibiting TRAIL-mediated apoptosis.

Multiple preclinical studies have demonstrated that AS1501 specifically binds to TRAIL, inhibits TRAIL-induced apoptosis, significantly reduces ALT and AST levels in liver injury and liver failure, markedly improves the prognosis of liver injury and liver failure caused by various etiologies, and shows potential to enhance ALT and AST recovery as well as long-term survival in patients with these conditions.

A single-center, randomized, double-blind, placebo-controlled, single-dose, dose-escalation study of AS1501 for injection has been conducted in healthy subjects. Among the 28 enrolled participants receiving the investigational drug, the doses of 1 mg/kg, 2 mg/kg, and 3 mg/kg were determined to be safe and well-tolerated.

Therefore, based on the nonclinical pharmacological and toxicological trial results of AS1501 and combined with Phase 1 clinical study data from healthy subjects, the sponsor conducted a

comprehensive evaluation of the benefit-risk profile and concluded that AS1501 offers potential clinical benefits with acceptable risks, supporting further clinical development in ACLF patients.

2. Study Objectives and End Points

2.1. test objective

2.1.1. Objective of Phase IIa Study

fundamental purpose :

- Assess the safety and tolerability of AS1501 for injection in early-stage ACLF patients, and explore an optimal dosing regimen.
- Determine the recommended dosage and administration frequency for Phase IIb studies.

Secondary objectives:

- Preliminary evaluation of the efficacy, immunogenicity, and pharmacokinetic (PK) characteristics of AS1501 for injection in patients with early acute lymphoblastic leukemia (ACLF).
- Preliminary investigation into the changes in serum TRAIL and NK cell membrane-type TRAIL expression levels before and after administration of AS1501 for injection in early-stage ACLF patients.

Exploratory Purpose:

- Preliminary investigation into the cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.

2.1.2. Objective of Phase IIb Study

fundamental purpose :

Assess the efficacy of AS1501 for injection in treating subjects with ACLF.

Secondary objectives:

- To evaluate the safety, pharmacokinetic (PK) characteristics, and immunogenicity of AS1501 for injection in patients with acute myeloid leukemia (AML).
- To investigate the changes in serum TRAIL levels and NK cell membrane-type TRAIL expression before and after administration of AS1501 for injection in ACLF patients.

Exploratory Purpose:

- Preliminary investigation into the cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.

2.2. End-point parameter of the trial

2.2.1. Stage IIa study endpoint

Primary endpoint:

- 1) The incidence rates of various adverse events (AEs) and serious adverse events (SAEs), as well as the incidence rates of AEs and SAEs associated with the study product.

Secondary endpoint:

- 1) Changes in MELD score and AARC score from baseline at the end of AS1501 treatment.
- 2) Survival rate of ACLF patients at the end of AS1501 treatment.
- 3) Improvement rate of ACLF patients at the end of AS1501 treatment; Criteria for improvement: ① Significant improvement in clinical symptoms such as fatigue, poor appetite, abdominal distension, and bleeding, with resolution of hepatic encephalopathy; ② Marked improvement in signs such as jaundice and ascites; ③ Significant improvement in liver function parameters (TBIL <5 ULN, PTA > 40%, or INR <1.5).
- 4) The proportion of ACLF patients with normal serum biochemical parameters (ALT, AST, Tbil, and PTA) at the end of AS1501 treatment.
- 5) The incidence of switching to other treatment modalities (artificial liver, liver transplantation, cell therapy, etc.).
- 6) Pharmacokinetic parameters of AS1501 after single and multiple administrations, including: apparent volume of distribution (V_d/f), plasma clearance (CL/F), plasma elimination half-life (t_{1/2}), peak plasma concentration (C_{max}), time to peak (T_{max}), area under the plasma concentration-time curve (AUC), steady-state peak plasma concentration (C_{ss-max}), steady-state time to peak (T_{ss-max}), steady-state trough plasma concentration (C_{ss-min}), area under the steady-state plasma concentration-time curve (AUC_{ss}), and accumulation ratio (R_{ac}).
- 7) Immunogenicity-related indicators, such as ADA antibodies.
- 8) Preliminary investigation into the changes in serum TRAIL and NK cell membrane-type TRAIL expression levels before and after administration of AS1501 for injection in ACLF patients.

Exploratory endpoints:

- 1) Preliminary investigation into cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.

2.2.2. Stage IIb study endpoint

Primary endpoint:

- 1) Survival rate of ACLF patients on day 28 after the first dose administration.

Secondary endpoint:

- 1) Survival rates of ACLF patients at weeks 8 and 12 after the first dose administration.
- 2) Improvement rates in ACLF patients at weeks 4, 8, and 12 after the first administration; criteria for improvement: ① Significant alleviation of clinical symptoms such as fatigue, poor appetite, abdominal distension, and bleeding, with resolution of hepatic encephalopathy; ② Marked improvement in signs such as jaundice and ascites; ③ Significant improvement in liver function parameters (TBIL <5 ULN, PTA > 40%, or INR <1.5).
- 3) The proportion of ACLF patients with normal serum biochemical response rates (ALT, AST, Tbil, PTA all within normal ranges) at weeks 4, 8, and 12 after the first dose administration.
- 4) Changes in the end-stage liver disease model (MELD) score and the Asian Association for Research on Liver Diseases ACLF Research Consortium (AARC) score of ACLF patients at weeks 4, 8, and 12 after the first administration compared to baseline.
- 5) The incidence of switching to other treatment modalities (artificial liver, liver transplantation, cell therapy, etc.).
- 6) Liver transplantation rate on day 90;
- 7) Pharmacokinetic parameters of AS1501 after multiple administrations, including steady-state plasma minimum concentration (C_{ss-min}).
- 8) Immunogenicity-related indicators, such as ADA antibodies.
- 9) The incidence rates of various adverse events (AEs) and serious adverse events (SAEs), as well as the incidence rates of AEs and SAEs associated with the study product.
- 10) Preliminary investigation into the changes in serum TRAIL and NK cell membrane-type TRAIL expression levels before and after administration of AS1501 for injection in ACLF patients.

Exploratory endpoints:

- 1) Preliminary investigation into cytokine release induced by AS1501 and the receptor saturation of AS1501 in vivo.

3. research design

3.1. system design

This trial is a Phase II clinical study conducted in two phases:

Phase IIa: A sentinel, single-arm study design is planned to enroll 12 early-stage ACLF patients receiving the 0.5 mg/kg dose regimen. The first two participants will serve as sentinels, enrolled sequentially and receiving a single intravenous dose. If these two sentinels do not experience any study drug-related serious adverse events (SAEs) within 2 weeks after the initial dose, the remaining 10 participants may be enrolled; otherwise, the dose will be reduced and the exploration phase continued. After the single intravenous dose, participants must undergo an 20-day washout period. During this washout period, if no study drug-related adverse events of grade ≥ 3 occur and safety and tolerability are jointly assessed by investigators and the sponsor, the participant proceeds to the multiple-dose phase (once weekly [with D21 being the first dose of multiple administration], for 4 consecutive weeks); otherwise, the dose will be reduced and the exploration phase continues, with the specific dose determined through joint discussion between the sponsor and investigators. If any participant drops out during the washout period after the single dose, additional participants may be recruited to ensure at least 12 subjects enter the multiple-dose phase.

After all 12 early-stage ACLF subjects in the 0.5 mg/kg dose group completed continuous dosing, the Safety Monitoring Committee (SMC) evaluated the safety profile of this dose group. The SMC would discuss whether to proceed with dose escalation if any of the following conditions occurred in the 0.5 mg/kg group:

- 1) At least one-third of the subjects experienced drug-related grade 3 adverse events (AEs).
- 2) Any drug-related adverse event (AE) of grade 4 or higher.

If, upon SMC evaluation, the subjects meet the dose escalation criteria, the remaining 12 early-stage ACLF participants eligible for enrollment will receive the 1 mg/kg dose group. Following the same enrollment rules as the 0.5 mg/kg dose group, the first two participants serve as sentinels and are enrolled sequentially for a single intravenous administration. If these two sentinels do not experience any study drug-related serious adverse events (SAEs) within 2 weeks after the initial dose, the remaining 10 participants may be enrolled; otherwise, the dose will be reduced to continue the exploratory study. After the single intravenous dose, subjects must undergo an 20-day washout period. During this washout period, if no study drug-related adverse events of grade ≥ 3 occur and both the investigator and the sponsor jointly assess safety and tolerability, the subject enters the multiple-dose

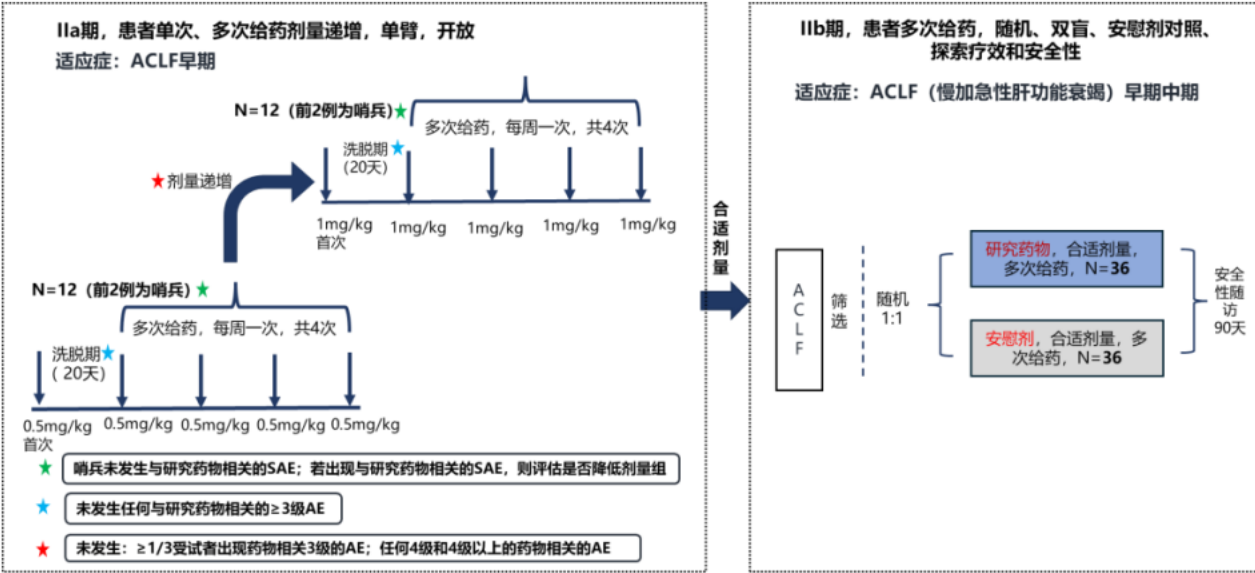
phase (once weekly [with D21 being the first dose of multiple administration], for 4 consecutive weeks). If any participant drops out during the washout period after the single dose, additional subjects may be recruited to ensure that at least 12 participants enter the multiple-dose phase.

Following completion of dose exploration studies at 0.5 mg/kg and 1 mg/kg, investigators and the sponsor may determine the recommended appropriate dose for Phase IIb trials based on cumulative safety data, efficacy results, and potential PK/PD findings; alternatively, they may explore other dose ranges or dosing frequencies.

Phase IIb: A randomized (1:1), double-blind, placebo-controlled parallel-design study was planned to enroll 72 ACLF patients receiving either appropriate doses and frequencies of AS1501 or placebo, with the aim of further evaluating the efficacy and safety of AS1501 for injection. The specific study design will be determined jointly by the investigators and the sponsor based on the results from Phase IIa.

The research process at each stage of this study is divided into the screening phase, treatment phase, and safety follow-up phase.

The specific design is shown in the figure below.



3.2. Dose-escalation design

3.2.1. Principle of Dose Incrementation at Termination

Phase IIa dose escalation

All 12 early-stage ACLF subjects in the first-dose group (0.5 mg/kg) had completed continuous dosing, after which the SMC evaluated the safety profile of this dose group. If any of the following

conditions occurred in the 0.5 mg/kg dose group, the SMC would discuss whether to proceed with dose escalation:

[1] At least one-third of the subjects experienced drug-related grade 3 adverse events (AEs).

[2] Any drug-related adverse event (AE) of grade 4 or higher;

determination of the Phase IIb dose

When all subjects receiving doses of 0.5 mg/kg or 1 mg/kg have completed treatment, the SMC will comprehensively determine the recommended dose for Phase IIb based on safety data (AEs, SAEs, AESIs), PK and PD parameters (TRAIL protein levels).

3.3. Definition of study completion

Stage IIa: The last participant completed the final visit.

Stage IIb: The last participant completes a 4-week efficacy and safety assessment or withdraws from the study early (whichever occurs first), and long-term follow-up data can be continuously collected.

Upon completion of the study in accordance with laws and regulations, provisions set by drug regulatory authorities, or requirements of the ethics committee, the sponsor shall report to the regulatory authority, and the sponsor or investigator shall report to the ethics committee.

3.4. Randomization and Blinding

This study IIb was designed as a randomized, double-blind trial.

The randomization numbers for participants were generated by an independent statistician unrelated to this study. Using the PLAN procedure in SAS 9.4, the independent statistician generated 72 random numbers via block randomization and assigned participants to either the AS1501 (test drug) group or the placebo group at a 1:1 ratio, with 36 subjects in each group. Researchers should assign random numbers in ascending order based on the chronological sequence of participant screening eligibility.

Blinding: Double-blind refers to a protocol where neither the subjects, investigators, monitors, nor data analysts are aware of the allocation of treatment drugs. The sponsor or its designated entity provides both the investigational drug and a placebo, ensuring that their appearance and weight are identical to those of the investigational drug. The sponsor-designated entity assigns blinding codes to both the investigational drug and placebo using a blinded randomization table provided by the statistician. These blind codes should be prepared in duplicate, sealed in envelopes, and retained separately by the clinical research lead institution and the drug regulatory applicant. Additionally, the statistician prepares an emergency unblinding envelope and sends it to the research center; in cases of serious adverse events (SAEs) or other emergencies, the study physician may open the corresponding

envelope for that subject to identify the assigned treatment drug for immediate intervention—a procedure known as emergency unblinding.

Blinding level: This trial employed a double-blind design, wherein neither the investigating physician nor the subjects were aware of which specific drug was administered to each participant.

The specific steps are as follows:

1) In accordance with GCP regulations regarding investigational drug management, all trial medications shall be packaged and labeled uniformly with the designation "for clinical trials only," ensuring complete consistency between the trial group packaging and that of the placebo. Blinding packaging shall be performed by designated personnel, with each subject receiving a separate package; the entire process must be verified by a designated individual and documented in detail. Randomization tables generated on-site after use shall be collected by the randomization statistician.

2) Upon distribution of the investigational drug to the study site, the site shall establish a dedicated location (drug storage area) for custody by designated personnel. The study site may appoint non-blind staff members, such as a non-blind medication dispensing nurse, as needed to ensure that both the investigating physicians and subjects remain in a blinded state.

Emergency Letters and Blind Baselines: Prior to the start of the trial, an emergency envelope is prepared for each subject, labeled with the subject's random number. The letter paper inside the envelope specifies the subject's group assignment, intended for emergency unblinding purposes. All investigational medications will be distributed to the study site along with the corresponding numbered emergency envelopes.

The blinding process shall be documented in writing and signed by all personnel involved in the blinding. The blinded record shall be sealed with an official stamp and stored separately by dedicated staff of the research institution and the sponsor; emergency unblinding letters shall be dispatched to the research institution along with the investigational drug and properly retained by dedicated staff of the research institution until the conclusion of the trial.

Emergency Declassification: When declassification is urgently required under emergency circumstances, the researcher must obtain approval from the head of the research unit. The classified document may be accessed only after obtaining written consent from the head. Within 24 hours following declassification, relevant personnel at the applying institution shall be notified with an explanation of the rationale for declassification. Emergency declassification may be considered in the following scenarios, including but not limited to:

(1) When a patient experiences a serious adverse event that may be associated with the trial drug or the control drug;

(2) When the patient develops severe complications.

Blinding removal protocol: This trial involves a single blinding removal procedure, which shall be conducted by the research institution and the sponsor after the database is closed. Personnel responsible for maintaining the blinded records shall submit the trial group assignments to the statistical department for analysis.

4. study population

4.1. Phase IIa study population

4.1.1. Eligibility criteria for Stage IIa

- [1] The age of the signatories to the informed consent form must range from 18 to 75 years old (including the boundary values).
- [2] During screening, chronic plus acute liver failure was diagnosed based on the Guidelines for Diagnosis and Treatment of Liver Failure (2018 Edition) issued by the Liver Failure and Artificial Liver Group of the Infectious Diseases Branch of the Chinese Medical Association and the Severe Liver Disease and Artificial Liver Group of the Hepatology Branch of the Chinese Medical Association. Patients were in the early stage of acute liver failure (ACLF) and did not meet the criteria for liver transplantation.
 - ◆ Early manifestations of ACLF:
 - Severe fatigue accompanied by significant gastrointestinal symptoms such as marked anorexia, vomiting, and abdominal distension;
 - ALT and/or AST levels remain significantly elevated, with progressive worsening of jaundice ($\text{TBil} \geq 171 \mu\text{mol/L}$ or daily increase $\geq 17.1 \mu\text{mol/L}$);
 - Hemorrhagic tendency is present in 30% of cases with PTA values ranging from $<30\%$ to $\leq 40\%$ (or INR values between 1.5 and <1.9).
 - No complications or other extrahepatic organ failures were observed.
- [3] During the screening phase, serum TRAIL levels are elevated to ≥ 2 times that of normal individuals, or NK cell membrane-type TRAIL is increased.
- [4] Can comprehend the informed consent form, voluntarily participate in it, and sign it;
- [5] Capable of completing the trial in accordance with the study protocol;

[6] Participants (including their partners) are willing to voluntarily adopt effective contraceptive measures from the screening stage through 6 months after the last administration of the investigational drug.

4.1.2. Exclusion Criteria for Stage IIa

Subjects are not eligible for inclusion in this study if any of the following criteria are met.

- [1] Patients with a history of allergic predisposition or severe hypersensitivity to protein-based drugs (CTCAE v5.0 grade>3);
- [2] Patients who have undergone liver transplantation or those planning to undergo liver transplantation within the next month.
- [3] ACLF patients at intermediate or advanced stages;
- [4] Severe Grade III ascites or refractory ascites;
- [5] Associated with stage III-IV hepatic encephalopathy.
- [6] Patients with concurrent malignant tumors or a history of malignant tumors; those diagnosed with lung cancer, liver cancer, pancreatic cancer, or gastrointestinal tumors through imaging studies (ultrasound, CT, or MRI) and tumor marker tests (e.g., AFP, CEA, CA125, or CA199) during the screening period or within one month prior to it.
- [7] Patients during the screening period or within 1 month prior to screening whose endoscopic or imaging (abdominal ultrasound, CT, or MRI) findings indicate severe varices with a bleeding risk.
- [8] The KDIGO criteria for acute kidney injury (AKI) define the following subjects: (1) a serum creatinine (Scr) level increase of $\geq 26.5 \mu\text{mol/L}$ (0.3 mg/dL ; $1 \text{ mg/dL} = 88.4 \mu\text{mol/L}$) within 48 hours; (2) a Scr elevation exceeding 1.5 times the baseline value within 7 days; and (3) reduced urine output ($<0.5 \text{ ml/kg/h}$) lasting more than 6 hours.
- [9] The following laboratory test values are abnormal: a. Complete blood count: platelets (PLT) $<75 \times 10^9/\text{L}$, hemoglobin (HGB) $<80 \text{ g/L}$; b. PT-INR ≥ 1.9 or PTA $\leq 30\%$; c. Left ventricular ejection fraction (LVEF) $<50\%$; serum creatinine $>1.5 \times \text{ULN}$.
- [10] Associated with severe respiratory dysfunction, dyspnea, or respiratory failure.
- [11] Severe infections that cannot be controlled by concomitant medications, including infections of major organs such as the abdominal cavity, lungs, urinary tract, and skin.
- [12] Individuals who are HIV-positive, or those with active pulmonary tuberculosis or syphilis infection.

- [13] Patients with a history of unstable ischemic heart disease, congestive heart failure, myocardial infarction (MI), stroke, severe arrhythmias, or other related conditions.
- [14] Patients with severe hypertension or diabetes that cannot be controlled with concomitant medications.
- [15] Women who are pregnant or breastfeeding, or those with a positive pregnancy test result.
- [16] Individuals who participated in clinical trials involving other drugs or medical devices within 30 days prior to randomization or during any of the five drug half-life periods.
- [17] The study subjects must have undergone trauma or major surgery (e.g., requiring general anesthesia) within 28 days prior to the first administration of the investigational drug. Note: Subjects scheduled for surgical procedures under local anesthesia are eligible to participate in the study.
- [18] Any serious underlying medical or psychiatric conditions (e.g., alcohol or drug abuse), dementia, or changes in mental status; or any issues that may impair the subject's ability to receive or tolerate the planned treatment at the research center, understand informed consent, or participate in the study, or that investigators consider contraindications for enrollment or potential interference with protocol-defined assessments or study outcomes.
- [19] The researchers identified other conditions that made participants unsuitable for this study.

4.2. Phase IIb study population

4.2.1. Stage IIb inclusion criteria

- [1] The age of the signatories to the informed consent form must range from 18 to 75 years old (including the boundary values).
- [2] The screening criteria were based on the "Guidelines for Diagnosis and Treatment of Liver Failure (2018 Edition)" issued by the Liver Failure and Artificial Liver Group of the Infectious Diseases Branch of the Chinese Medical Association and the Severe Liver Disease and Artificial Liver Group of the Hepatology Branch of the Chinese Medical Association, requiring a diagnosis of chronic plus acute liver failure at an early or intermediate stage of ACLF (Acute Complicated Liver Failure) and failure to meet the eligibility criteria for liver transplantation.
 - ◆ Early manifestations of liver failure:
 - Severe fatigue accompanied by significant gastrointestinal symptoms such as marked anorexia, vomiting, and abdominal distension;

- ALT and/or AST levels remain significantly elevated, with progressive worsening of jaundice (TBil $\geq 171 \mu\text{mol/L}$ or daily increase $\geq 17.1 \mu\text{mol/L}$);
- Hemorrhagic tendency is present in 30% of cases with PTA values ranging from $<30\%$ to $\leq 40\%$ (or INR values between 1.5 and <1.9).
- No complications or other extrahepatic organ failures were observed.
- ◆ Mid-stage manifestations of liver failure:
 - Based on the early manifestations of liver failure, the condition further progresses.
 - ALT and/or AST levels decline rapidly, while TBil continues to rise.
 - The bleeding is evident (with petechiae or ecchymoses), with $20\% < \text{PTA} \leq 30\%$ (or $1.9 \leq \text{INR} < 2.6$).
 - Associated with one complication and/or one extrahepatic organ failure.

- [3] During the screening phase, serum TRAIL levels are elevated to ≥ 2 times that of normal individuals, or NK cell membrane-type TRAIL is increased.
- [4] Can comprehend the informed consent form, voluntarily participate in it, and sign it;
- [5] Capable of completing the trial in accordance with the study protocol;
- [6] Participants (including their partners) are willing to voluntarily adopt effective contraceptive measures from the screening stage through 6 months after the last administration of the investigational drug.

4.2.2. Exclusion Criteria for Stage IIb

Subjects are not eligible for inclusion in this study if any of the following criteria are met.

- [1] Patients with a history of allergic predisposition or severe hypersensitivity to protein-based drugs (CTCAE v5.0 grade >3);
- [2] Patients who have undergone liver transplantation or those planning to undergo liver transplantation within the next month.
- [3] Advanced-stage ACLF patients.
- [4] Severe Grade III ascites or refractory ascites.
- [5] Associated with stage III-IV hepatic encephalopathy.
- [6] Patients with concurrent malignant tumors or a history of malignant tumors; those diagnosed with lung cancer, liver cancer, pancreatic cancer, or gastrointestinal tumors through imaging studies

(ultrasound, CT, or MRI) and tumor marker tests (e.g., AFP, CEA, CA125, or CA199) during the screening period or within one month prior to it.

- [7] Patients during the screening period or within 1 month prior to screening whose endoscopic or imaging (abdominal ultrasound, CT, or MRI) findings indicate severe varices with a bleeding risk.
- [8] Patients with severe coagulation failure defined as PT-INR ≥ 2.6 or PTA $\leq 20\%$; platelet count (PLT) $<75 \times 10^9/L$; and hemoglobin (HGB) <80 g/L.
- [9] Associated with severe respiratory dysfunction, dyspnea, or respiratory failure.
- [10] Severe infections that cannot be controlled by concomitant medications, including infections of major organs such as the abdominal cavity, lungs, urinary tract, and skin.
- [11] Individuals who are HIV-positive, or those with active pulmonary tuberculosis or syphilis infection.
- [12] Patients with a history of unstable ischemic heart disease, congestive heart failure, myocardial infarction (MI), stroke, severe arrhythmias, or other related conditions.
- [13] Patients with severe hypertension or diabetes that cannot be controlled with concomitant medications.
- [14] Women who are pregnant or breastfeeding, or those with a positive pregnancy test result.
- [15] Individuals who participated in clinical trials involving other drugs or medical devices within 30 days prior to randomization or during any of the five drug half-life periods.
- [16] The study subjects must have undergone trauma or major surgery (e.g., requiring general anesthesia) within 28 days prior to the first administration of the investigational drug. Note: Subjects scheduled for surgical procedures under local anesthesia are eligible to participate in the study.
- [17] Any serious underlying medical or psychiatric conditions (e.g., alcohol or drug abuse), dementia, or changes in mental status; or any issues that may impair the subject's ability to receive or tolerate the planned treatment at the research center, understand informed consent, or participate in the study, or that investigators consider contraindications for enrollment or potential interference with protocol-defined assessments or study outcomes.
- [18] The researchers identified other conditions that made participants unsuitable for this study.

4.3. Lifestyle Considerations

Throughout the entire trial, participants are required to:

- During the 24-hour period preceding each administration cycle and throughout the collection of final pharmacokinetic (PK) and/or pharmacodynamic samples, consumption of products containing caffeine or xanthines (e.g., coffee, tea, cola, and chocolate) is

prohibited.

- Alcohol consumption is prohibited for 24 hours prior to each administration cycle until the collection of final pharmacokinetic (PK) and/or pharmacodynamic samples.
- Smokers are prohibited from using any nicotine-containing products (including nicotine patches) at the research center.
- Intense physical activity is prohibited within 8 hours prior to each blood draw for laboratory tests. Participants may engage in light recreational activities during the procedure (e.g., watching television, reading).
- Avoid sexual intercourse with individuals of the opposite sex who have immunodeficiency whenever possible.

4.4. Subject Allocation and Numbering

All participants enrolled in the screening will receive a unique participant number. This number serves as a fixed identifier for each participant throughout the study. The participant number consists of five digits: the first two represent the center identification number, and the remaining three are sequentially assigned by the research center during screening.

4.5. Screening failed

A screening failure is defined as a situation where a subject has signed an informed consent form but was unable to receive the study drug due to certain reasons, such as failing to meet inclusion criteria or meeting at least one exclusion criterion. For subjects with screening failures, it is essential to collect at least the following basic information: the date of informed consent signing, demographic characteristics, the reason for screening failure, and all serious adverse events (SAEs) occurring between the signing of the Informed Consent Form (ICF) and the occurrence of screening failure.

Abnormal results from screening-related tests (e.g., complete blood count, blood biochemistry, urinalysis, coagulation function tests, 12-ECG, etc.) may be retested during the screening period if objective evidence indicates that the underlying cause has changed or if the investigator determines it to be a transient abnormality; successful retesting qualifies participants for enrollment.

Subjects determined to have failed screening (based on laboratory evaluation results and other assessments) may undergo re-screening once, provided they sign the Informed Consent Form (ICF) again. Each subject is eligible for a maximum of one additional re-screening opportunity.

4.6. Subject Replacement

If a subject withdraws from the study prior to receiving drug therapy, an additional subject must be enrolled. If a subject discontinues participation after initiating drug therapy, no replacement subject is

required. However, in Phase IIa, if a subject drops out during the elution period following a single dose administration, a replacement subject may be included to ensure that at least 12 subjects progress to the multiple-dose phase.

4.7. Study discontinuation/termination; subject withdrawal from the study

4.7.1. Study interruption/termination

Recruitment of subjects will be suspended under any of the following circumstances:

- 1) The principal investigator, sponsor, and statistical experts concluded that the number and severity of adverse events warranted early termination of the study.
- 2) During the implementation of the trial, significant deviations or human errors were identified, severely compromising trial quality and preventing the achievement of intended objectives.
- 3) After joint evaluation by the investigators and the sponsor, the study drug therapy was deemed ineffective.
- 4) Newly diagnosed malignant tumors were detected in subjects after AS1501 infusion, and these were considered potentially related to AS1501.
- 5) The competent administrative authority has ordered the termination of the trial;
- 6) Under the premise of fully safeguarding the subjects' rights and safety, the sponsor requests to terminate the trial (e.g., due to financial or administrative reasons).
- 7) Other circumstances that require suspension of recruitment, as jointly assessed by the investigators and the sponsor.

If there are sufficient valid reasons, this study may be temporarily suspended or terminated early.

If the study is temporarily suspended or terminated early, the principal investigator shall promptly notify the participants and the ethics review committee, providing reasons for the termination or suspension. Where appropriate, the investigator will contact the participants to inform them of any changes to the visit schedule. The study may only proceed further after issues related to safety, protocol adherence, and data quality have been resolved and with the consent of the clinical trial site and/or regulatory authorities.

When the study intervention is discontinued, subsequent research procedures shall be completed in accordance with the trial protocol. If a subject exhibits clinically significant changes (including but not limited to deviations from baseline levels) after enrollment, the investigator or a qualified designated personnel shall determine whether adjustments to the subject management are required. Any clinically relevant new findings shall be reported as adverse events (AEs).

4.7.2. Subjects discontinued or withdrew from the study

Subjects may request to terminate or withdraw from the study at any time, and all reasons must be recorded in the Case Report Form (CRF), including but not limited to the following:

The subject voluntarily withdrew from the study.

The subject withdrew their informed consent.

encyesis ;

Lost to follow-up;

die ;

other ;

The subjects selected by the researchers were discontinued or withdrawn from the study.

Significant protocol deviations or violations occurred during the study, which substantially impacted drug tolerance, safety, or pharmacokinetic (PK) evaluation.

From a medical ethics perspective, the researchers deemed it necessary to terminate the trial for patients requiring urgent liver transplantation who have suitable donor liver sources available.

Patients who experienced life-threatening serious adverse events (SAEs) related to the investigational drug and are not suitable for continuing participation in the trial;

After discontinuing the study drug therapy, the researchers determined that withdrawal from the study was most beneficial for the subject.

Poor subject compliance (e.g., failure to adhere to prescribed medication regimens, examination protocols, or use of other drugs that may affect efficacy and safety evaluation outcomes); severe protocol violations/deviations;

The occurrence of a clinical adverse event (AE), laboratory abnormalities, or other medical conditions may pose a risk to the subject's safety or health if continued participation in the study is warranted, and would not maximize the subject's benefit.

Subjects meeting the exclusion criteria (newly identified or previously undetected) are not eligible to continue participating in the study.

Subjects who have signed the informed consent form and undergone randomization but have not received the study intervention can be replaced.

4.7.3. Treatment Completion Criteria

Subjects may request discontinuation of treatment at any time, and all reasons must be recorded in the Case Report Form (CRF), including but not limited to the following:

- The subject withdrew their informed consent.
- Unbearable toxicity occurs;
- Poor compliance;
- The subject requests to terminate treatment (e.g., considering consideration of artificial liver support);
- Lost to follow-up;
- die ;
- encyesis ;
- other 。

4.7.4. Lost to follow-up

If a subject fails to return as scheduled multiple times or the research center is unable to contact the subject, the subject will be considered lost to follow-up.

If the subject fails to return to the research center for the required study visit, the following measures must be taken:

- The research center must attempt to contact the subjects, reschedule any missed visits as soon as possible, explain the importance of adhering to the scheduled visit schedule, and determine whether the subjects are willing and/or should continue participating in the study.
- Before confirming that a subject has become lost to follow-up, the investigator or designated personnel must make every effort to recontact the subject (performing up to three telephone calls where possible; if necessary, sending registered mail or equivalent methods to the subject's last known mailing address). All such attempts shall be documented in the subject's medical records.
- If the subject cannot be contacted after the aforementioned attempts, they are considered to have withdrawn from the study.

5. Research Intervention

5.1. research on drug

5.1.1. Research Drug Description

AS1501, a investigational drug developed by Shenzhen Zhongke Aishen Pharmaceutical Co., Ltd., is a Class 1 therapeutic biologic that competitively binds to TRAIL-R2 (DR5) on cell surfaces for the tumor necrosis factor-related apoptosis-inducing ligand (TRAL).

The detailed specifications of AS1501 are shown in the figure below.

Chinese Common Name	AS1501 for Injection
English Generic Name	AS1501 for Injection
active ingredient	Human recombinant AS1501 fusion protein
Excipients	Sodium dihydrogen phosphate monohydrate, Disodium hydrogen phosphate dihydrate, Sodium chloride, Mannitol, Polysorbate 20
Structure Formula	The dimeric fusion protein consists of the extracellular domain fragment of truncated human death receptor 5 (DeathReceptor5) combined with the Fc segment (including the hinge, CH2, and CH3 regions) of human IgG1.
formula weight	Approximately 79 KD
physical characteristics	The drug has a pH of 6.7 – 7.3; an osmotic pressure molar concentration of 270 – 330 mOsmol/kg; and moisture content not exceeding 3.0%.
stability	The validity period is tentatively set at 3 years
the form of a drug	Lyophilized Powder for Injection
shape and properties	White loose bodies
specifications	25 mg per vial
storage procedures	Seal tightly, store away from light, refrigerated at 2°C–8°C
Administration Method	Intravenous infusion
Administration Frequency	Stage IIa: a total of 5 sessions. Stage IIb: tentatively scheduled once weekly, a total of 4 sessions.

In Phase IIb, the placebo was a white lyophilized powder injection with identical appearance, administered via the same route as AS1501.

5.1.2. Study of drug administration regimens

medication

Administration is performed via intravenous infusion: AS1501 for injection is reconstituted in sterile water for injection and then mixed with 0.9% sodium chloride injection (the total volume of the test drug, sterile water for injection, and 0.9% sodium chloride injection is 200 mL). The infusion duration should be no less than 2 hours. During Phase IIb, placebo administration follows the same protocol as that for AS1501.

Pre-treatment before administration

In the Phase 1 clinical study of AS1501 conducted in healthy volunteers, two cases of grade 3 hypersensitivity reactions were observed in the 2 mg/kg dose group, and two cases of CRS were reported in the 3 mg/kg dose group. Therefore, in this study, if any dose group in Phase IIa exhibits ≥ grade 2

hypersensitivity reactions or infusion reactions related to the investigational drug, or if CRS of any severity is observed, the affected subject and other subjects enrolled subsequently may receive pretreatment prior to administration of the investigational drug. This pretreatment should include at least glucocorticoids (dexamethasone 5–10 mg or equivalent agents). Additionally, anti-histamines (oral or intravenous diphenhydramine 25 mg–50 mg) and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg) may be administered as per standard protocols at each center.

Number of doses/frequency

The initial dosing regimen is tentatively scheduled every 7 days, with Phase IIa planned to involve 5 doses (including a single initial dose and 4 consecutive doses). For Phase IIb, the dosage regimen is tentatively set at once weekly for a total of 4 doses. The specific dosing schedule (including number of doses and frequency) will be determined comprehensively based on the data from Phase IIa.

5.1.3. Expected toxic management measures and dose adjustment principles

5.1.3.1. Phase IIa single-dose administration during the elution period

During the single-dose infusion phase, if any grade ≥ 3 adverse event (AE) related to the study drug occurs in a subject, active supportive therapy shall be administered as per institutional protocols until the AE resolves to grade < 2 or returns to baseline levels. The investigator must conduct a thorough evaluation; if continued administration is deemed beneficial, the subject will proceed to the continuous dosing phase. If the subject cannot receive continuous dosing as scheduled on Day 21, dose delay may be considered for up to 2 weeks, with subsequent doses administered at n+7, n+14, and n+21 based on the actual administration date (n).

5.1.3.2. Multiple-dose administration phase

For Phase IIa subjects entering the multiple-dose administration phase and Phase IIb subjects, AS1501 dosage adjustments may be made based on the severity of adverse reactions. Toxicity in subjects should be assessed using the NCI CTCAE v5.0 grading scale. The primary management strategy for toxicity (at any level) involves dose adjustment or delayed administration of the study drug. Any modification to the dosing regimen must be discussed with the sponsor's medical monitor prior to implementation, unless there is an immediate safety concern, and shall be supervised by qualified medical personnel at the research site (the principal investigator or assistant investigator).

- a) Treatment may be suspended for up to 3 weeks (calculated from the next scheduled administration time) until toxicity returns to a level permitting continued therapy or baseline

values. Treatment may only be resumed after more than 3 weeks if clear clinical benefit is observed and approved by the sponsor's medical monitor. After reducing the study drug dose, no higher dose level shall be increased without prior approval from the sponsor's medical monitor.

- b) Treatment will be discontinued if toxicity meets the criteria for treatment termination.
- c) During the administration process, if the subject suspends or delays drug administration and subsequently resumes it, the study medication shall continue to be administered at subsequent dates (e.g., n+7, n+14, or n+21, as applicable) starting from the actual resumption date (n).

For dosage adjustment principles, refer to the table below:

AE Grade (CTCAE)	Dose Adjustment Principles
Level 1 or Level 2	
AE can occur any number of times	According to the investigators' assessment, if the subject can tolerate the toxicity, the maintenance dose will remain unchanged.
Grade 3, and associated with the investigational drug	
AE occurs any number of times	Discontinue administration until toxicity returns to baseline levels or reaches grade 1 or tolerable grade 2. In the high-dose group, resume dosing after reducing the dose level; in the low-dose group, terminate treatment or resume administration at the investigator's discretion.
Grade 4, and associated with the investigational drug	
AE occurs any number of times	The investigator shall determine whether to terminate treatment. If resumption of administration is decided, it should be initiated at one dose level lower in the high-dose group only after toxicity has returned to baseline levels or resolved to grade 1 or tolerable grade 2. For the low-dose group, the decision to explore lower doses may be made jointly by the sponsor and the investigator.

5.1.3.3. Expected Toxicity Mitigation Measures

5.1.3.3.1. lymphocytopenia

Lymphocytopenia has been observed in Phase I studies involving healthy subjects; the recommended management measures are as follows:

toxicity criterion	measure
≤Level 2	○ Perform regular complete blood count (CBC) tests until remission is achieved at grade ≤1 or baseline levels. ○ Continue follow-up until remission or return to baseline levels; ○ Provide active supportive therapy;
Grade ≥3	○ On hold treatment; ○ Perform regular complete blood count (CBC) tests until remission is achieved at grade ≤1 or baseline levels. ○ If no improvement is observed, continue follow-up; ○ Provide active supportive therapy;

toxicity criterion	measure
	○ According to the researchers' assessment, treatment can be resumed at a reduced dose level after symptom relief.

5.1.3.3.2. Hypersensitivity reactions, infusion reactions

In the Phase I clinical study of this investigational drug conducted in healthy volunteers, two cases of grade 3 hypersensitivity reactions were observed in the 2 mg/kg dose group across two instances. If any dose group in Phase IIa reports $\geq$ grade 2 hypersensitivity reactions or infusion-related reactions associated with the investigational drug, both the affected subject and other subjects enrolled subsequently may receive anti-allergic treatment prior to drug administration, including: glucocorticoids (dexamethasone 5–10 mg or equivalent), antihistamines (oral or intravenous diphenhydramine 25 mg or 50 mg), and antipyretic analgesics (oral acetaminophen 650 mg or 1000 mg). The specific dosing regimen shall be determined by the investigator in accordance with institutional protocols.

In the event of hypersensitivity reactions or infusion reactions, the recommended management measures are as follows:

toxicity criterion	measure
$\leq$ Level 2	○ Continue follow-up until remission is achieved or the condition stabilizes to grade ≤ 1; ○ Reduce the infusion rate or suspend the infusion; ○ Provide active supportive therapy with glucocorticoids (dexamethasone 5 – 10 mg or equivalent agents), antihistamines (oral or intravenous diphenhydramine 25 mg – 50 mg), and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg), while maintaining close monitoring. If symptoms improve, administer AS1501 again at 50% of the original infusion rate; if a grade ≥ 3 infusion reaction occurs again, discontinue AS1501 permanently. ○ Following each administration, anti-allergic drugs should be administered. If a grade ≥ 3 infusion reaction occurs again, AS1501 should be discontinued permanently.
Level 3	○ On hold treatment; ○ Continue follow-up until remission or return to baseline levels; ○ Provide active supportive therapy with glucocorticoids (dexamethasone 5 – 10 mg or equivalent agents), antihistamines (oral or intravenous diphenhydramine 25 mg – 50 mg), and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg), while maintaining close monitoring. If symptoms improve, administer AS1501 again at 50% of the original

toxicity criterion	measure
	infusion rate; if a grade ≥ 3 infusion reaction occurs again, discontinue AS1501 permanently. Following each administration, anti-allergic drugs should be administered. If a grade ≥ 3 infusion reaction occurs again, AS1501 should be discontinued permanently.
Grade ≥ 4	- Permanent discontinuation of medication; - Continue follow-up until remission or return to baseline levels; - Administer active treatment: glucocorticoids (dexamethasone 5–10 mg or equivalent agents), antihistamines (oral or intravenous diphenhydramine 25 mg – 50 mg), and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg), followed by close monitoring.

5.1.3.3.3. Cytokine Release Syndrome

Cytokine Release Syndrome (CRS) is a hyperphysiological response characterized by the activation of endogenous or infused T cells and/or other immune effector cells following immunotherapy. It manifests as the activation and release of large numbers of lymphocytes (B cells, T cells, and/or natural killer cells) and/or bone marrow cells (macrophages, dendritic cells, and monocytes), leading to the production of inflammatory cytokines such as IFN- γ , TNF- α , IL-6, and IL-10. Among these, IL-6 is associated with the severity of CRS [42].

Mild symptoms of CRS include fever, fatigue, headache, rash, arthralgia, and myalgia. More severe manifestations involve hypotension and high fever, which may progress to an uncontrolled systemic inflammatory response accompanied by circulatory shock requiring vasopressors, vascular leakage, disseminated intravascular coagulation (DIC), and multi-organ system failure.

CRS should be highly suspected if any of the following symptoms or signs that cannot be explained by other causes occur after AS1501 infusion: ① fever $\geq 38^{\circ}\text{C}$; ② hypotension (defined as systolic blood pressure <90 mmHg, below baseline value); ③ hypoxemia (requiring oxygen therapy to correct oxygenation deficiency).

In the event of CRS, the recommended management measures are as follows:

CRS classify	Treatment Recommendations
Level 1	- Reduce the infusion rate or suspend the infusion; - Continue follow-up until remission is achieved or the condition stabilizes to grade ≤ 1; - Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to control body temperature;

	- 排除 potential infectious pathogens (by blood, urine, and sputum cultures; pulmonary imaging studies, etc.). - Broad-spectrum antibiotics should be administered in cases of combined agranulocytosis; - If refractory fever persists after symptomatic treatment (body temperature >39°C for more than 10 hours or persistent fever lasting over 3 days), dexamethasone 5–10 mg intravenously may be considered. - According to the researchers' assessment, treatment may be resumed at the original dose or reduced by one dose level after symptom relief.
Level 2	- Reduce the infusion rate or suspend the infusion; - Continue follow-up until remission is achieved or the condition stabilizes to grade ≤1; - Based on the aforementioned Level 1 CRS clinical management protocol, administer additional fluid therapy with 10–20 ml/kg of normal saline; repeat fluid administration if necessary to maintain blood pressure. - Dexamethasone 10 mg intravenously every 6 hours (or equivalent dose), or methylprednisolone 1 mg/kg intravenously every 6 hours; - Low-flow oxygen therapy support; - According to the researchers' assessment, treatment may be resumed at the original dose or reduced by one dose level after symptom relief.
Level 3	- On hold treatment; - Symptomatic and supportive management (fever reduction, fluid replacement, maintenance of internal homeostasis, etc.); - Administer rapid infusion fluids via an on-demand intravenous pump and use vasopressors as needed. - Dexamethasone 10 – 20 mg intravenously every 6 hours (or equivalent dose), or methylprednisolone 1–2 mg/kg intravenously every 6 hours. - If the aforementioned treatments prove ineffective or if contraindications to hormonal therapy exist, plasma exchange may be considered. - Administer high-flow oxygen therapy; - If necessary, consider transferring the patient to the ICU and perform hemodynamic monitoring. - According to the researchers' assessment, treatment can be resumed at a reduced dose level after symptom relief.
Level 4	- Permanent discontinuation of medication; - The patient should be transferred to the ICU for enhanced monitoring and treatment. - Dexamethasone 20 mg intravenously every 6 hours (or equivalent dose) or methylprednisolone 500 mg intravenously every 12 hours, administered continuously for 3 days; the dosage should be rapidly tapered after symptom control. - Symptomatic and supportive management (fever reduction, fluid replacement, maintenance of internal homeostasis, etc.); - Provide mechanical ventilation as needed; - Administer rapid infusion fluids via an on-demand intravenous pump and utilize various vasoactive agents; - ICU monitoring and hemodynamic surveillance; - If the aforementioned treatments prove ineffective, plasma exchange may be considered.

Note: For patients receiving hormone therapy for CRS, the investigator may evaluate whether prophylactic antifungal medication should be administered. Dexamethasone/methylprednisolone may be substituted with other corticosteroids at equivalent doses.

5.2. Preparation/processing/storing responsibilities for investigational drugs

5.2.1. Reception and inventory of medications

The sponsor is responsible for delivering the investigational drug (AS1501 or placebo) to the research center, with the investigator/pharmacist signing to confirm receipt.

The investigational drug (AS1501 or placebo) may only be used for this study and must be managed exclusively by personnel authorized by the investigator. The investigator/pharmacist responsible for drug management shall distribute, collect, and destroy the investigational drugs in accordance with the study protocol, while maintaining accurate records.

5.2.2. Product Storage and Stability

The investigator or their designated personnel must ensure that all investigational drugs are sealed, protected from light, and stored at a temperature of 2°C to 8°C during transportation. In case of any deviations, they must report the situation and address the issues prior to initiating the study treatment.

Only enrolled study subjects may receive the investigational treatment, and only authorized personnel at the research center may administer or manage the investigational drug. All investigational drugs or related items must be stored under the conditions specified on the labels in a secure, environment-controlled, and monitorable (manual or automated) area, with access restricted solely to investigators and authorized research center personnel.

The principal investigator, institution, or head of a medical facility (as applicable) is responsible for accountability, coordination, and record maintenance regarding the study treatment (i.e., receiving, coordinating, and ultimately disposing of records).

5.3. Randomization and Blinding

Study IIb was designed as a randomized, double-blind trial. Detailed descriptions are provided in Section 4.4.

5.4. Research on intervention adherence

Treatment adherence is defined as the ratio of the actual number of medications administered to subjects during the study period to the prescribed number of medications. Throughout the study, all medication administration under the study regimen will be recorded in the Electronic Data Collection

(EDC) system. Any deviations from the protocol-specified medication regimen will be documented, including the administration date and rationale.

Researchers will assess the subjects' adherence to the study treatment at each visit.

5.5. Concomitant Medications and Treatment

5.5.1. Prohibited Medications and Treatments

During the trial, the use of artificial liver support systems, glucocorticoids (excluding those used for pretreatment or to manage adverse events such as infusion reactions/cytokine release syndrome), hepatocyte growth factors, and any drugs with confirmed hepatotoxicity (including traditional Chinese medicines and proprietary Chinese formulations) is prohibited. Additionally, plasma, cold precipitates, and other blood products must not be administered within 48 hours prior to AARC or MELD score assessments.

5.5.2. permitted medications and treatments

Combined Therapy with ACLF

ACLF participants are permitted to receive standard therapeutic medications, including but not limited to necessary nutritional support, plasma transfusion (excluding within 48 hours prior to AARC or MELD score assessment), platelet transfusion, albumin supplementation, intravenous catheterization, dynamic monitoring, fluid resuscitation, microbiota modulation, analgesics, sedatives, antibiotics, oxygen therapy, and mechanical ventilation.

Pre-treatment therapy

If grade ≥ 2 drug-related hypersensitivity reactions, infusion reactions, or CRS of any severity are observed in any dose group at stage IIa, the affected subject and all subsequent enrolled subjects may receive pretreatment therapy prior to study drug administration, including at least glucocorticoids (dexamethasone 5 – 10 mg or equivalent). Additionally, anti-histamines (oral or intravenous diphenhydramine 25 mg–50 mg) and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg) may be administered as per institutional protocols.

Treatment for AE

Subjects are permitted to receive symptomatic supportive treatment for adverse events (AEs), including but not limited to anti-infective therapy, nutritional support, fluid replacement, and blood transfusion. If AEs such as infusion reactions or cytokine release syndrome occur after administration of the investigational drug, treatments may include corticosteroids (dexamethasone 5–10 mg or equivalent agents), antihistamines (oral or intravenous

diphenhydramine 25 mg–50 mg), and antipyretic analgesics (oral acetaminophen 650 mg–1000 mg).

6. stages of research

6.1. Screening Period

6.1.1. Stage IIa screening period

In principle, all participants must sign an informed consent form approved by the Ethics Committee (IEC) prior to commencing the study-related examinations.

Researchers must explain to participants the medical evaluation prior to study initiation, potential risks and benefits, and emphasize that participation in this study is voluntary. They must provide participants with a written informed consent form signed by the researcher and dated accordingly.

ACLF patients must complete the following examinations and evaluations prior to enrollment:

- 1) Sign the informed consent form;
- 2) Collect demographic data;
- 3) Inquire about medical history/surgical history, current medications/complications, and personal history;
- 4) Perform a comprehensive physical examination;
- 5) Measure height (cm), weight (kg), and BMI;
- 6) Monitor vital signs;
- 7) 12-lead electrocardiogram;
- 8) Imaging studies: abdominal CT (for the digestive system), echocardiography;
- 9) Clinical laboratory tests:
 - HCG blood pregnancy test (for women of childbearing age);
 - Serological virological testing;
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - routine urine test ;
 - arterial blood gas analysis ;
 - Inflammatory markers;
- 10) Disease severity score:

- Child-Pugh score;
- The American Association for the Study of Liver Disease (AARC) scoring system for acute liver failure (ACLF);
- MELD score for end-stage liver disease models;
- SIRS diagnose ;

11) Clinical symptom assessment;

12) Selection and exclusion criteria determination.

13) Special examination: TRAIL blood sample.

14) adverse event 。

Participants must meet all inclusion criteria to participate in this study, and all screening procedures must be completed within 14 days after signing the informed consent form.

6.1.2. Phase IIb screening period

In principle, all participants must sign an informed consent form approved by the Ethics Committee (IEC) prior to commencing the study-related examinations.

Researchers must explain to participants the medical evaluation prior to study initiation, potential risks and benefits, and emphasize that participation in this study is voluntary. They must provide participants with a written informed consent form signed by the researcher and dated accordingly.

ACLF patients must complete the following examinations and evaluations prior to enrollment:

- 1) Sign the informed consent form;
- 2) Collect demographic data;
- 3) Inquire about medical history/surgical history, current medications/complications, and personal history;
- 4) Perform a comprehensive physical examination;
- 5) Measure height (cm), weight (kg), and BMI;
- 6) Monitor vital signs;
- 7) 12-lead electrocardiogram;
- 8) Imaging studies: abdominal CT (for the digestive system), echocardiography;
- 9) Clinical laboratory tests:
 - HCG blood pregnancy test (for women of childbearing age);

- Serological virological testing;
- Complete blood count (CBC);
- Blood biochemical tests;
- Coagulation function tests;
- blood gas analysis ;
- Urinalysis;
- Inflammatory markers.

10) Disease severity score:

- AARC grade ;
- MELD grade ;
- Child-Pugh score;
- SIRS diagnose ;

11) Clinical symptom assessment;

12) Selection and exclusion criteria determination.

13) Special examination: TRAIL blood sample.

14) adverse event 。

Subjects must meet all inclusion criteria to participate in this study, and all screening procedures must be completed within 14 days after signing the informed consent form. Upon selection, patients will be randomly assigned to either the AS1501 group or the placebo treatment group for combined therapy in addition to standard treatment regimens.

6.2. stage of therapy

6.2.1. Treatment period for stage IIa

For all hospitalization days from Day 1 to Day 42, the subjects were required to complete the following baseline items:

- 1) Basic treatments permitted by the protocol;
- 2) Record of concomitant medication use;
- 3) Clinical symptom assessment;
- 4) Adverse Event Record.

During the study treatment period, the items to be completed on the day of treatment evaluation and/or blood sampling are described as follows.

Day 1 (First administration)

Prior to and after the initial administration of the study drug, subjects must complete the following items:

- 1) Basic therapy and concomitant medications;
- 2) Weight measurement before the first administration;
- 3) check-up ;
- 4) Life signs: within 0.5 hours 10 1 minute before administration; at 1 hour 10 1 minute, 2 hours 10 1 minute, and 4 hours 20 1 minute after infusion initiation.
- 5) 12-lead electrocardiogram: within 0.5 h 10 1 min before administration; at 0 h $\pm$ 10 min, 1 h 10 1 min, 2 h 10 1 min, and 8 h 20 min after D1 infusion completion.
- 6) Laboratory tests (results within 3 days prior to study drug administration are acceptable):
 - HCG blood pregnancy test (for women of childbearing age only);
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - Urinalysis;
 - arterial blood gas analysis ;
 - Inflammatory markers;
- 7) Disease severity score (evaluated within 3 days prior to administration of the study drug):
 - Child-Pugh score;
 - The American Association for the Study of Liver Disease (AARC) scoring system for acute liver failure (ACLF);
 - MELD score for end-stage liver disease models;
 - SIRS diagnose ;

- 8) Cytokines: collected prior to the first administration (0.5 h 5 min) and at 0 hours post-infusion completion (± 30 minutes).
- 9) Immunogenicity: within 0.5 hours 5 1 minute prior to the first administration;
- 10) PK: Within 0.5 hours before the first administration, during the 5 minute prior to administration; at 1 hour ± 5 minutes after infusion initiation; at 0 hours ± 5 minutes, 1 hour ± 5 minutes, 2 hours ± 5 minutes, and 8 hours ± 10 minutes after completion of the entire infusion.
- 11) Cytokine release induced by AS1501 and receptor saturation of AS1501 in vivo: Collection time points are the same as those for PK studies.
- 12) PD biomarker (TRAIL): measured before the first dose administration (within 0.5 h 5 1 min) and at 0 hours ± 5 minutes post-infusion completion.
- 13) Investigate pharmacological treatments;
- 14) adverse event .

Days 2 to 14 (elution period after the first administration)

During the elution period following the first administration, the examinations and evaluations required of the subjects include:

- 1) Basic therapy and concomitant medications;
- 2) Life signs: at 144 hours (Day 7) and 312 hours (Day 14) after infusion completion;
- 3) 12-lead electrocardiogram: at 144 hours (Day 7) and 312 hours (Day 14) after infusion completion;
- 4) Laboratory tests: 144 hours (Day 7) and 312 hours (Day 14) after infusion completion.
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - Urinalysis;
- 5) Cytokines: Collected 24 hours (± 30 minutes) after infusion completion (Day 2).

6) Immunogenicity: measured at 144 ± 30 min post-infusion after administration on Day 1

(D7) and at 312 ± 30 min post-administration on Day 14 (D14).

7) PK: At $24 \text{ h} \pm 30 \text{ min}$ (D2), $72 \text{ h} \pm 30 \text{ min}$ (D4), $144 \text{ h} \pm 30 \text{ min}$ (D7), and $312 \text{ h} \pm 30 \text{ min}$ (D14) after infusion completion;

8) Cytokine release induced by AS1501 and receptor saturation of AS1501 in vivo: Collection time points are the same as those for PK studies.

9) PD biomarker (TRAIL): measured at 144 ± 30 min (Day 7) and 312 ± 30 min (Day 14) after infusion completion.

10) adverse event .

Days 21 to 42 (continuous administration periods: D21, D28, D35, D42)

During the continuous drug administration period of the study, subjects are required to complete the following items:

- 1) Basic therapy and concomitant medications;
- 2) Weight: before administration at D21/D28/D35/D42;
- 3) Physical examination: before administration of D21/D28/D35/D42;
- 4) Life signs: measured $0.5 \text{ h} \pm 10 \text{ min}$ before administration on days D21/D28/D35/D42, and at $1 \text{ h} \pm 10 \text{ min}$, $2 \text{ h} \pm 10 \text{ min}$, and $4 \text{ h} \pm 20 \text{ min}$ after infusion initiation.
- 5) 12-lead electrocardiogram (ECG): before drug administration (within $0.5 \text{ h} \pm 10 \text{ min}$) at D21/D28/D35/D42; at $0 \text{ h} \pm 10 \text{ min}$, $1 \text{ h} \pm 10 \text{ min}$, $2 \text{ h} \pm 10 \text{ min}$, and $8 \text{ h} \pm 20 \text{ min}$ after completion of infusion at these time points; if abnormalities such as QTcF interval prolongation occur, investigators may increase the examination frequency based on the subject's condition; post-administration, investigators may decide to perform additional ECG examinations.
- 6) Laboratory tests (to be completed within 24 hours prior to study drug administration on days D21, D28, D35, or D42):
 - Complete blood count (CBC);

- Blood biochemical tests;
 - Coagulation function tests;
 - Urinalysis;
 - arterial blood gas analysis
- 7) Assessment of disease severity (to be completed within 24 hours prior to study drug administration on D21/D28/D35/D42):
- AARC grade ;
 - MELD grade ;
 - Child-Pugh score;
 - Is SIRS present?
- 8) Cytokines: D21/D28/D35/D42 were collected before each infusion start (0.5 h $\pm$ 5 min), at 0 hours ($\pm$ 30 min) after each infusion, and at 24 hours ($\pm$ 30 min) post-infusion.
- 9) Immunogenicity: administered before D21/D28/D35/D42 (0.5 h $\pm$ 5 min);
- 10) Blood samples were collected at the following time points: before administration of PK D21/D28/D35 (within 0.5 h $\pm$ 5 min), before administration of PK D42 (within 0.5 h $\pm$ 5 min); and at 1 h $\pm$ 5 min after infusion initiation, 0 h $\pm$ 5 min after infusion completion, 1 h $\pm$ 5 min after infusion completion, 2 h $\pm$ 5 min after infusion completion, 8 h $\pm$ 10 min after infusion completion, 24 h $\pm$ 30 min after infusion completion (D43), 72 h $\pm$ 30 min after infusion completion (D45), 144 h $\pm$ 30 min after infusion completion (D48), and 312 h $\pm$ 30 min after infusion completion (D55).
- 11) Cytokine release induced by AS1501 and receptor saturation of AS1501 in vivo: Collection time points are the same as those for PK studies.
- 12) PD biomarker (TRAIL): before D21/D28/D35 administration (within 0.5 h $\pm$ 5 min), before infusion at D42 (fourth consecutive administration) (within 0.5 h $\pm$ 5 min), and at 0 h $\pm$ 5 min, 144 h $\pm$ 30 min (day 48), and 312 h $\pm$ 30 min (D55) after infusion completion.

13) Investigate pharmacological treatments;

14) adverse event .

6.2.2. Treatment period for stage IIb

For all hospitalization days from Day 1 to Day 28, the subjects were required to complete the following baseline items:

- 1) Basic treatments permitted by the protocol;
- 2) Record of concomitant medication use;
- 3) vital sign ;
- 4) Clinical symptom assessment;
- 5) Adverse Event Record.

During the study treatment period, the items to be completed on the day of treatment evaluation and/or blood sampling are described as follows.

Day 1 to Day 28 (provisionally administered weekly: D1/D8/D15/D22, for a total of 4 doses)

During the drug administration study, subjects must complete the following items:

- 1) Basic therapy and concomitant medications;
- 2) Patients were randomized before D1 administration;
- 3) Measuring body weight before each administration of D1/D8/D15/D22 and on day D28;
- 4) Physical examination: before each administration on days D1/D8/D15/D22, and on day D28;
- 5) Life signs: On days D1, D8, D15, and D22, within 0.5 hours 10 1 minute before each administration; at 1 hour 10 1 minute, 2 hours 10 1 minute, 4 hours 20 1 minute after infusion initiation; and on day D28.
- 6) 12-lead electrocardiogram: D1/D8/D15/D22; measurements should be taken within 0.5 h 10 1 min before each administration, and at the following time points after each infusion: 0 h $\pm$ 10 min, 1 h 10 1 min, 2 h 10 1 min, 8 h 20 min, and D28.
- 7) Laboratory tests (to be completed before administration on D1/D8/D15/D22 and on D28):
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;

- Urinalysis;
 - arterial blood gas analysis ;
 - Blood pregnancy test (before D1 administration, limited to women of childbearing age; results within 3 days are acceptable).
 - Inflammatory markers: Not required before D1 administration (if the screening examination is within ≤ 3 days).
- 7) Assessment of disease severity (to be completed before administration on D1/D8/D15/D22 and at D28):
- Child-Pugh score;
 - AARC grade ;
 - MELD grade ;
 - Is SIRS present?
- 8) Cytokines: Samples were collected before each infusion (within 0.5 h $\pm$ 5 min), at 0 hours ($\pm$ 30 min) after each infusion, and at 24 hours ($\pm$ 30 min) following each infusion.
- 9) Immunogenicity: Before D1/D8/D15/D22 administration (within 0.5 h $\pm$ 5 min);
- 10) Pharmacokinetic (PK) blood sampling: On days D8, D15, and D22, PK blood samples at the trough concentration should be collected before infusion (within 0.5 h $\pm$ 5 min); on days D1 and D22, PK blood samples at the peak concentration should be collected immediately after infusion completion (at 0 h $\pm$ 5 min).
- 11) Cytokine release induced by AS1501 and receptor saturation of AS1501 in vivo: Collection time points are the same as those for PK studies.
- 12) Collection of PD biomarker (TRAIL blood sample): before administration on days D1, D8, D15, and D22 (within 0.5 h $\pm$ 5 min).
- 13) Pharmacological study: AS1501 and placebo were administered to subjects at time points D1, D8, D15, and D22, respectively.
- 14) Clinical symptom assessment;

15) adverse event .

6.3. follow-up period

6.3.1. Follow-up period for Stage IIa

End the follow-up visit

Subjects who withdraw early or have completed four consecutive dosing sessions are required to undergo a treatment completion visit. The following items must be completed:

- 1) Basic therapy and concomitant medications;
- 2) vital sign ;
- 3) check-up ;
- 4) Measure body weight (kg);
- 5) 12-lead electrocardiogram;
- 6) Laboratory Testing Items:
 - HCG blood pregnancy test (for women of childbearing age);
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - Urinalysis;
 - arterial blood gas analysis ;
 - Inflammatory markers;
- 6) Assessment of disease severity:
 - Child-Pugh score;
 - AARC grade ;
 - MELD grade ;
 - Is SIRS present?
- 7) immunogenicity ;
- 8) adverse event ;

9) survival condition .

6.3.2. Follow-up period for Stage IIb

End the follow-up visit

Subjects who withdraw early or have completed four consecutive dosing sessions are required to undergo a treatment completion visit. The following items must be completed:

- 1) Basic therapy and concomitant medications;
- 2) weight ;
- 3) vital sign ;
- 4) check-up ;
- 5) 12-lead electrocardiogram;
- 6) Laboratory Testing Items:
 - Blood pregnancy test (for women of childbearing age)
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - blood gas analysis ;
 - Urinalysis;
- 7) Assessment of disease severity:
 - Child-Pugh score;
 - AARC grade ;
 - MELD grade ;
 - Is SIRS present?
- 7) PD (TRAIL blood sample collection);
- 8) adverse event ;
- 9) Clinical symptom assessment;
- 10) Record of survival status and liver transplantation status.

Day 60 of 7

Subjects who withdraw early or have completed four consecutive dosing sessions are required to undergo a D60 visit and complete the following items:

- 1) Basic therapy and concomitant medications;
- 2) weight ;
- 3) vital sign ;
- 4) 12-lead electrocardiogram;
- 5) Laboratory Testing Items:
 - Complete blood count (CBC);
 - Blood biochemical tests;
 - Coagulation function tests;
 - blood gas analysis ;
 - Urinalysis;
- 6) Assessment of disease severity:
 - Child-Pugh score;
 - AARC grade ;
 - MELD grade ;
 - Is SIRS present?
- 7) Clinical symptom assessment;
- 8) Record of survival status and liver transplantation status.

Day 90 7

Subjects who withdraw early or have completed four consecutive dosing sessions are required to undergo a D90 visit and complete the following items:

- 1) Basic therapy and concomitant medications;
- 2) weight ;
- 3) vital sign ;
- 4) 12-lead electrocardiogram;

5) Laboratory Testing Items:

- Complete blood count (CBC);
- Blood biochemical tests;
- Coagulation function tests;
- blood gas analysis ;
- Urinalysis;

6) Assessment of disease severity:

- Child-Pugh score;
- AARC grade ;
- MELD grade ;
- Is SIRS present?

7) Clinical symptom assessment;

8) Record of survival status and liver transplantation status.

6.4. Sample collection, processing, storage, and testing

6.4.1. Blood sample collection and pretreatment

PK sample collection: Collect whole venous blood into a sampling tube, gently invert and mix after completion. Allow the tube to stand upright at room temperature for at least 30 minutes. Subsequently, centrifuge at 1800–2200 g for 10 minutes at 2–8°C. Immediately collect the upper serum layer and evenly distribute it into labeled cryovials.

PD Sample Collection: The primary objectives are to measure serum TRAIL levels and the membrane-bound TRAIL content of NK cells. Collect whole venous blood into a collection tube, gently invert and mix after sampling. Allow the tube to stand upright at room temperature for at least 30 minutes. Then centrifuge at 1800–2200 g for 10 minutes at 2–8°C. Immediately collect the supernatant serum and aliquot it evenly into labeled cryovials. Concurrently, collect whole blood for NK cell TRAIL detection.

Immunogenicity (ADA) Sample Collection: Collect whole blood from a vein into an anticoagulation-free blood collection tube. After collection, gently invert and mix the sample. Allow the tube to stand upright at room temperature for at least 30 minutes. Subsequently, centrifuge the sample at 1800–2200 g for 10 minutes at 2–8°C. Immediately collect the upper serum layer and evenly

distribute it into labeled cryovials.

After serum distribution, tightly seal the tube and place it with the cap opening facing upward into a cryovessel. Store the cryovessel vertically in a refrigerator at -60°C to -90°C until transportation to the bioanalytical laboratory. Each sample is assigned a unique sample number. The interval between whole blood collection and storage in the refrigerator must not exceed 2 hours. Test samples are shipped to the analysis laboratory under dry ice conditions, while backup samples are retained at the clinical center.

The actual date and time of blood sample collection, as well as the precise administration time, shall be recorded in the original medical record and on the PK, PD, and ADA blood sampling pages of the eCRF. Any issues encountered during blood sampling shall be noted in both the original medical record and the eCRF.

6.4.2. Sample Tube Information Label

Before blood sample collection, the blood collection tubes and cryovials shall be uniformly numbered and labeled with dedicated tags.

The label must include the following information: study protocol number, subject number, dose group details, sample type (PK, PD, immunogenicity), collection time point, and sample category (test sample, backup sample).

6.4.3. Storage and Transportation of Samples

The biological samples for testing are transported via cold chain to the testing facility, with temperature maintained between -60 °C and -90 °C throughout transit. Comprehensive temperature monitoring is implemented, accompanied by transportation temperature records, a list of biological samples, and a sample transfer form.

For biological samples exhibiting abnormal characteristics during clinical trials that do not require testing, the clinical institution shall provide relevant documentation. Upon receipt of such samples, the institution must inspect the sample identification, condition, and quantity, record the acceptance details, and retain a handover list provided by the clinical trial unit containing information on sample identification, status, quantity, source, transportation method and conditions, and transport duration. The testing unit shall retain temperature monitoring data from the cold chain transportation process and store the biological samples in refrigerators with temperature control ranging from -60°C to -90°C.

All specific requirements for blood sample collection and processing must comply with the standard operating procedures of the central laboratory regarding sample collection, processing, storage,

and transportation.

6.4.4. Sample Testing

(1) PK Sample Testing:

PK blood sampling time points

Stage IIa: Blood samples were collected at the following time points: before infusion initiation on D1 (within 0.5 h $\pm$ 5 min), 1 h $\pm$ 5 min after infusion start on D1, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, and 24 h $\pm$ 30 min after infusion completion (D2); 72 h $\pm$ 30 min (D4); 144 h $\pm$ 30 min (D7); 312 h $\pm$ 30 min (D14); prior to dosing on D21/D28/D35 (within 0.5 h $\pm$ 5 min); prior to dosing on D42 (within 0.5 h $\pm$ 5 min); and again at 1 h $\pm$ 5 min after infusion start, 0 h $\pm$ 5 min, 1 h $\pm$ 5 min, 2 h $\pm$ 5 min, 8 h $\pm$ 10 min, and 24 h $\pm$ 30 min after infusion completion (D43); 72 h $\pm$ 30 min (D45); 144 h $\pm$ 30 min (D48); and 312 h $\pm$ 30 min (D55). A total of 23 PK blood sampling time points were tentatively designated.

Phase IIb: Subjects underwent collection of plasma samples at trough concentration PK measurements before infusion on days D8, D15, and D22 (within 0.5 h $\pm$ 5 min), and at peak concentration PK measurements immediately after infusion completion on days D1 and D22 (at 0 h $\pm$ 5 min). A total of five PK sampling time points were provisionally designated.

PK evaluating indicator

The mean values at each time point were calculated based on individual plasma drug concentrations, and individual or average plasma drug concentration-time curves were plotted. Pharmacokinetic (PK) parameters related to the drug were statistically analyzed, including peak concentration (C_{max}), time to peak concentration (T_{max}), area under the plasma drug concentration-time curve (AUC), mean retention time (MRT), apparent volume of distribution (V_d), total clearance rate (CL/F), and volume of distribution (V_z/F).

(2) PD Sample Testing:

PD Blood Collection Time Point

Stage IIa: Screening phase, with blood sampling at 12 PD time points: before D1 infusion (within 0.5 h $\pm$ 5 min), at 0 h $\pm$ 5 min after D1 infusion completion, at 144 h $\pm$ 30 min (D7), at 312 h $\pm$ 30 min

(D14), before administration on D21/D28/D35 (within 0.5 h $\pm$ 5 min), before D42 infusion (fourth consecutive administration) (within 0.5 h $\pm$ 5 min), and at 0 h $\pm$ 5 min and 144 h $\pm$ 30 min after D42 infusion completion (D48), as well as at 312 h $\pm$ 30 min after D55 infusion.

Stage IIb: Screening phase; before administration of D1/D8/D15/D22 (within 0.5 h $\pm$ 5 min); and at the end-of-treatment visit, TRAIL blood samples were collected at six designated time points in total.

PD Biomarkers

Biomarkers primarily include the investigational drug target protein TRAIL.

(3) Immunological Sample Testing

Stage IIa: Before D1 administration, at 144 $\pm$ 30 min post-D1 administration (D7), at 312 $\pm$ 30 min post-D1 administration (D14), before administrations on D21, D28, D35, and D42 (within 0.5 $\pm$ 5 min), and during the final treatment visit; immunogenic blood samples were collected at these eight designated time points.

Stage IIb: Before administration of D1/D8/D15/D22 (within 0.5 h $\pm$ 5 min). During the trial, the subsequent immunogenicity sampling points may be adjusted based on preliminary immunogenicity test results.

The immunogenicity (ADA) assay was performed by a third-party bioanalytical laboratory designated by the sponsor. The testing methodology was validated prior to implementation, and the analytical results were reported independently without inclusion in the study summary report.

7. Research Evaluation

7.1. Demographics and other baseline characteristics

Demographic statistics and baseline characteristic variables will be summarized by dose group. Continuous variables (e.g., age, height, weight) will be summarized using descriptive statistics, while categorical variables (e.g., gender, ethnicity) will be summarized as frequencies and percentages. Separate summaries will be performed for each analysis set.

7.2. PK, PD estimate

7.2.1. Pharmacokinetic Evaluation

For each subject receiving the study drug, pharmacokinetic parameters were determined based on the plasma concentration-time curve.

Main PK parameters: Cmax, Tmax, t1/2, CL, AUC0-t, AUC0-inf, RAUC, RCmax, etc.

Other PK parameters: CL/F (total clearance), λ_z (terminal rate constant), Vz/F (distribution volume), Vss (steady-state distribution volume), etc.

7.2.2. PD Parameter Evaluation

For each subject receiving the study drug, pharmacokinetic parameters were determined based on serum TRAIL levels and NK cell membrane-type TRAIL levels before and after administration.

7.2.3. Group PK/PD Evaluation

Based on data characteristics and the drug's mechanism of action, select an appropriate model for population PK/PD or PPK studies to identify covariates influencing PK/PD.

7.3. Efficacy Evaluation

Phase IIa efficacy analysis

The efficacy evaluation primarily assesses collected laboratory parameters—including blood biochemistry, complete blood count, coagulation function, and blood gas analysis—after four consecutive dosing administrations, while evaluating changes in TBil, PT-INR or PTA, AST/ALT, ALB, Pre-ALB, Scr, LA, MELD score, Child-Pugh score, AARC score, SIRS diagnosis, and infection-related indicators.

Effectiveness evaluation primarily includes the following key indicators:

Multiple dosing period (total of 4 administrations, with D21 as the first administration) evaluation

- Baseline: All test results prior to D21 administration
- Changes in MELD score, AARC score, and Child-Pugh score from baseline to the end of treatment;
- Survival rate at the end of treatment;
- Changes in TBil, DBil, PT-INR, ALB, Pre-ALB, AST/ALT, serum creatinine, and serum lactate levels from baseline to the end of treatment;
- Improvement rate of ACLF patients at the end of AS1501 treatment;
- The proportion of ACLF patients with normal serum biochemical parameters (ALT, AST, Tbil, PTA) at the end of AS1501 treatment;

Phase IIb efficacy analysis:

The efficacy evaluation primarily focuses on assessing efficacy data within 28 days post-first administration, supplemented by efficacy data collected during the 90-day follow-up period. Data collection methods included recording mortality or liver transplantation occurrences within 28 days after randomization, as well as monitoring complications related to liver failure. Blood biochemical parameters, complete blood count (CBC), and coagulation function tests were collected during the screening phase, within 24 hours prior to each administration, and on day 28; changes in TBil, PT-INR/PTA, AST/ALT, ALB, Pre-ALB, Scr, LA, MELD score, Child-Pugh score, AARC score, SIRS diagnosis, and infection-related indicators were also evaluated. Additionally, key clinical biochemical markers, AARC scores, survival status, liver transplantation outcomes, and mortality rates were assessed at days 60 and 90 of the follow-up period.

Effectiveness evaluation primarily includes the following key indicators:

- Baseline: All scoring results and test results prior to D1 administration;
- Changes in the AARC-ACLF score from baseline to day 28 after the first dose administration;
- Survival rate on day 28 after the first dose;
- Survival rates of ACLF patients at weeks 8 and 12 after the first administration;
- Improvement rates in ACLF patients at weeks 4, 8, and 12 after the first administration;
- The proportion of ACLF patients with normal serum biochemical response rates (ALT, AST, Tbil, PTA all within normal ranges) at weeks 4, 8, and 12 after the first dose administration;
- Changes in the end-stage liver disease model (MELD) score and the Asian Association for Research on Liver Diseases ACLF Research Alliance (AARC) score of ACLF patients at weeks 4, 8, and 12 after the first administration compared to baseline;
- The incidence of switching to other treatment modalities (artificial liver, liver transplantation, cell therapy, etc.);
- 90-day liver transplantation rate;

MELD score for end-stage liver disease models

The End-Stage Liver Disease Model (MELD) is a system primarily utilizing serum bilirubin, international normalized ratio of prothrombin time, and serum creatinine levels to assess end-stage liver disease. The MELD score predicts mortality in end-stage liver disease and is widely employed in evaluations of liver transplantation, severe hepatitis, and hepatocellular carcinoma. This study adopted its modified formula: $MELD = 3.8 \times \ln[TBil \text{ (mg/dL)}] + 11.2 \times \ln[INR] + 9.6 \times \ln[Scr \text{ (mg/dL)}] +$

6.4 × (0 for cholestatic or alcoholic liver disease; 1 for other etiologies), with results rounded to whole numbers. Detailed scoring criteria are provided in Appendix 5.

Child-Pugh classification criteria

The Child-Pugh classification system is a clinically widely used grading standard for quantitatively assessing hepatic reserve function in patients with cirrhosis. First proposed by Child in 1964, this system categorizes five patient parameters—including general condition, ascites, serum bilirubin, serum albumin concentration, and prothrombin time—into three levels assigned scores of 1, 2, or 3 respectively. The total score is calculated by summing these five parameters, ranging from a minimum of 5 to a maximum of 15 points, which corresponds to three grades of hepatic reserve impairment (Grade A, B, or C), indicating varying degrees of liver dysfunction (higher scores indicate poorer hepatic reserve function). Detailed scoring criteria are provided in Appendix 3.

Asia-Pacific Association for Research on Liver Diseases (AARC) scoring system

In 2017, the Asia-Pacific Association for Liver Diseases (APASL) enrolled 1,402 patients with acute liver failure (ALF) meeting APASL criteria. The study identified bilirubin levels, hepatic encephalopathy severity scores, international normalized ratio (INR), lactate levels, and creatinine concentrations as prognostic factors, leading to the development of the ALF-AARC score. This scoring system ranges from 5 to 15 points across three grades: Grade I (5–7 points), Grade II (8–10 points), and Grade III (11–15 points), corresponding to 28-day mortality rates of 12.7%, 44.5%, and 85.9%, respectively. The AARC score is straightforward to calculate, utilizes readily available clinical parameters, and enables dynamic monitoring of patient prognosis. Detailed scoring criteria are provided in Appendix 4.

7.4. Safety and Other Evaluations

的生命 signs, physical examination, laboratory tests (complete blood count, blood biochemistry, urinalysis, coagulation function), and 12-lead electrocardiogram; adverse events and serious adverse events.

vital sign

的生命体征 examination (body temperature, pulse, respiration, blood pressure) will be performed

at the time points specified in the study flowchart and trial protocol.

Reference ranges for vital signs: systolic blood pressure 90–140 mmHg, diastolic blood pressure 60–90 mmHg (in sitting position).

Pulse rate: 60–100 beats per minute

Breath rate: 12–20 times per minute

Body temperature ranges from 35.8 to 37.2 °C (forehead temperature, including boundary values)

check-up

The physical examination (general condition, head, neck, lymph nodes, chest, abdomen, musculoskeletal system, skin, nervous system, and other areas) will be performed at the time points specified in the study flowchart.

12-lead electrocardiogram

For each subject, a 12-lead electrocardiogram (ECG) will be obtained at the time points specified in the study flowchart. All ECG examinations must be performed after the subject has rested for at least 5 minutes. During ECG acquisition, the subject must maintain a supine position and remain conscious throughout the procedure.

laboratory examination

The following laboratory tests will be performed at the time points specified in the study flowchart.

Table 1: Laboratory Test Items

Laboratory Evaluation	parameter
routine blood test	White blood cell count (WBC), Neutrophil count (ANC), Neutrophil percentage, Eosinophil percentage, Basophil percentage, Lymphocyte count (LYM), Lymphocyte percentage, Monocyte count (MONO), Monocyte percentage, Red blood cell count (RBC), Hemoglobin (HGB), Platelet count (PLT)
Blood Biochemistry	Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin (ALB), pre-albumin (Pre-ALB), total bilirubin (TBIL), direct bilirubin (DBIL), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT),

	creatinine (CREA), urea (UREA), uric acid (UA), potassium (K), sodium (Na), chloride (Cl), calcium (Ca), magnesium (Mg), phosphorus (P), glucose, lactate dehydrogenase (LDH), creatine kinase, and uric acid.
coagulation function	Prothrombin time (PT), International Normalized Ratio (INR), Activated partial thromboplastin time (APTT), Fibrinogen (FIB), Prothrombin activity (PTA).
Virus Screening	Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), hepatitis C antibody (Anti-HCV), hepatitis B virus DNA (HBV-DNA), hepatitis C virus RNA (HCV-RNA), human immunodeficiency virus antibody (HIV), Treponema pallidum antibody.
URAN	pH, glucose, protein, occult blood, ketones, bilirubin, urobilinogen, nitrite, white blood cells, red blood cells, specific gravity.
Inflammatory markers	C-reactive protein (CRP), procalcitonin, etc.
arterial blood gas analysis	Acid-base balance (pH), oxygen partial pressure (PO ₂), arterial oxygen saturation (SaO ₂), carbon dioxide partial pressure (PCO ₂), lactate, etc.
pregnancy tests	Blood pregnancy tests are required only for female subjects of childbearing age during the screening period, prior to the first administration, and at the end-of-treatment visit. Additional serum pregnancy tests may be performed by investigators based on clinical indications.
cell factor	At least TNF- α and IL-6 must be included; researchers may add other cytokine assays based on the available cytokine testing panels at each center, taking into account the specific characteristics of the study subjects.
Cytokine release induced by AS1501, and receptor saturation of AS1501 in vivo	Collect peripheral blood cells to measure the cytokine release induced by AS1501 (including IL-2, IFN- γ , IL-6, TNF- α , IL-10, etc.) and assess the receptor saturation of AS1501 in vivo.

1. Adverse Events and Serious Adverse Events

1.1. adverse event

1.1.1. Definition of Adverse Events

Adverse events (AEs) refer to all adverse medical events occurring in subjects after administration of the investigational drug, which may manifest as symptoms, signs, diseases, or abnormal laboratory findings, but not necessarily have a causal relationship with the investigational drug.

AEs include, but are not limited to: (1) exacerbation of pre-existing medical conditions/diseases (including worsening of symptoms, signs, or abnormal laboratory findings); (2) onset of any new adverse medical condition (including symptoms, signs, or newly diagnosed diseases); (3) clinically significant abnormal laboratory test values

1.1.2. Severity of adverse events

Grading refers to the severity of adverse events (AEs). The severity of AEs is determined according to the CTCAE 5.0 criteria established by the U.S. Department of Health and Human Services. For AEs not listed in the table, their severity should be graded based on Table 17.

Table 1: Severity of Adverse Events

grade	CTCAE Status
1	Mild: Asymptomatic or mild; observed solely clinically or diagnostically; requires no treatment.
2	Moderate: Requires minor, localized, or non-invasive treatment; accompanied by age-appropriate limitations in instrumental activities of daily living*.
3	Severe or medically significant but not immediately life-threatening: leads to hospitalization or prolonged hospital stay; causes disability; restricts independent daily living activities.
4	Life-threatening: requires emergency treatment.
5	Death related to adverse events.

CTCAE = General Terminology Standard for Adverse Events

*: Instrumental daily activities include cooking, shopping for clothing, using the telephone, and managing finances.

**Self-directed daily activities refer to tasks such as bathing, dressing/dressing off, eating, personal hygiene, and taking medication, without requiring bed rest.

1.1.3. Assessment of the correlation between adverse events and the medication

The causal relationship assessment between AE and the study treatment in this study will employ a

Table 1 Relationship between adverse events and the study drug

	Definitely relevant	Highly likely	May be related	May be irrelevant	Definitely unrelated
THE STUDY DRUGS SHOULD BE ADMINISTERED IN A RATIONAL CHRONOLOGICAL ORDER.	+	+	+	+	-
KNOWN TYPES OF DRUG REACTIONS	+	+	+	-	-
THE REACTION DIMINISHES OR DISAPPEARS AFTER DISCONTINUATION OF THE MEDICATION.	+	+	±	±	-
THE ADVERSE REACTIONS RECURRED AFTER REPEATED ADMINISTRATION.	+	?	?	?	-
CANNOT BE EXPLAINED BY THE SUBJECT'S DISEASE	+	+	-	±	-

Note: + indicates affirmation, -indicates negation, ± indicates possibility or uncertainty

○ causal relationship between adverse events and the investigational drug

The causal relationship between adverse events and the investigational drug should be assessed by the investigator based on all available information when completing the eCRF.

For all adverse events (AEs), investigators should separately evaluate the causal relationship between each event and each investigational drug. If investigators determine that an event cannot be unequivocally attributed to a specific investigational drug (e.g., suspected potential interaction), the same evaluation should be recorded for each treatment regimen. The assessment of causality shall be conducted by authorized clinicians, who must not only determine whether a causal relationship exists with the investigational drug but also provide as detailed a rationale as possible.

Key factors to consider when evaluating the causal relationship between adverse events and the investigational drug include:

- a. Regarding the temporal relationship with administration: The event should occur after drug administration. The duration from drug exposure to the occurrence of the event should be evaluated within its clinical context.
- b. Reactions after discontinuation of medication (deactivation) and after re-administration (reactivation): The patient's response following deactivation or reactivation should be evaluated based on the typical clinical course of the relevant condition.
- c. Underlying diseases, comorbidities, and complications: Each event should be evaluated in conjunction with the current treatment regimen and the natural history and progression of any other potential diseases the patient may have.
- d. Concomitant medications or treatments: The other drugs or treatments the patient is receiving at the time of the adverse event should be reviewed to determine whether they may have contributed to the event.
- e. Known types of reactions to such drugs (clinical/preclinical)
- f. Physiological/psychological stress exposure: Stress exposure may induce adverse changes in patients and provide a more plausible explanation for the observed phenomenon.
- g. Pharmacological and pharmacokinetic studies of the investigational treatment: The pharmacokinetic characteristics (absorption, distribution, metabolism, and excretion) of the investigational treatment as well as its pharmacodynamics in individual patients should be comprehensively evaluated.

○ Assessment of the correlation between adverse events and the medication

The combined categories of "definitely related," "highly likely related," and "possibly related" were defined as adverse reactions to the investigational drug, and the incidence rate of adverse reactions was calculated accordingly.

The classification of determination results and the criteria for determination are summarized in Table 18.

special explanation :

- a. The evaluation of adverse events for their drug-relatedness shall be conducted by personnel with relevant medical expertise.
- b. Table 18 may not cover all scenarios encountered in actual practice. When the criteria presented in Table 18 do not fully align, reference the professional judgment logic for assessing drug-related adverse events provided in the table to arrive at the most reasonable conclusion possible.

c. When additional information and evidence regarding the association between adverse events and the drug are obtained during clinical trial implementation, any modification to previously established correlation conclusions should be supported by sufficient rationale.

1.1.4. Collection of adverse events

When collecting adverse event information from subjects, a consistent, non-invasive questioning approach should be employed. Adverse event (AE) collection begins upon the subject's signing of the informed consent form and continues until the end of the safety follow-up period (28 days $\pm$ 7 days after the last dose) or until the subject initiates new liver protection therapy (e.g., artificial liver support or liver transplantation). Investigators must monitor the patient's health status throughout the study. Clinical adverse events occurring between the signing of the informed consent form and before the first administration of the drug are recorded as part of the medical history/complications section in the Case Report Form (CRF) and are not classified as AEs unless one of the following conditions is met:

- Any injury/damage resulting from clinical laboratory testing procedures;
- Adverse events caused by treatment discontinuation related to the trial protocol;
- Adverse events caused by medications other than the investigational drug administered as part of the treatment regimen.

After the safety follow-up period (28 days $\pm$ 7 days post-last dose) concludes, no further active collection of adverse events (AEs) will be performed. However, if investigators identify serious adverse events (SAEs) with a well-established causal relationship to the investigational drug, these must still be reported to the sponsor (or the CRO designated by the sponsor).

The sources of AE include:

- The patient's responses to questions regarding their health status (standard, non-guided questions are posed during each visit, such as "How have you been feeling since your last visit?").
- Symptoms reported spontaneously by the patient.
- The researchers concluded that their findings represent clinically significant changes or abnormalities detected during examination.
- Other patient health-related information known to the researchers (e.g., hospitalization).

1.1.5. Record of adverse events

Researchers must record all adverse events in the adverse event section provided in each patient's eCRF, which should include the following information:

- Name of Adverse Event
- Start date and time (you can omit the time if applicable)
- order of severity
- Relationship to the investigational drug
- Measures taken for investigational drugs
- Other measures taken
- Date and time of outcome (you may omit the time if applicable)
- Outcomes
- Whether it constitutes a serious adverse event and the corresponding severity criteria

➤ **Name of the adverse event**

The name of an adverse event should be a medical term, with preference given to medical diagnoses. If a definitive diagnosis cannot be established, individual symptoms or signs should be recorded separately; the diagnosis should be updated upon subsequent confirmation, replacing the previous symptoms or signs. If the same adverse event occurs more than once in a patient and the patient recovers between episodes, each occurrence should be recorded separately.

It must be noted that the measures taken do not constitute adverse events; rather, the reasons for taking those measures are the adverse events. Hospitalization itself is not an adverse event; the underlying reason for hospitalization constitutes the adverse event. Death does not qualify as an adverse event; instead, the cause of death represents the adverse event (sudden death of unknown origin may be recorded as "death of unknown cause").

➤ **Measures taken for investigational drugs**

The measures taken regarding the investigational drug to address adverse events must be classified as one of the following:

- Permanent discontinuation – The investigational drug is permanently discontinued due to specific adverse events (AEs).
- No dose adjustment required – No specific adverse event (AE) necessitates modification of the trial drug regimen; continue administration as usual.
- Dose reduction – the dosage is decreased due to specific adverse events (AEs).
- Suspension of medication – temporarily discontinuing (including voluntary

discontinuation by the subject) the investigational drug due to specific adverse events (AEs), followed by subsequent resumption of administration.

- Not applicable – discontinuation of the investigational drug for reasons other than specific adverse events (AEs), such as termination of the study, subject death, or prior discontinuation of the investigational drug before the occurrence of an AE.

➤ **Other measures taken**

Adverse events requiring treatment must be managed according to established medical standards to safeguard patient health and interests. Corresponding cardiopulmonary resuscitation (CPR) equipment and medications must be readily available to ensure optimal treatment can be provided in emergency situations.

If pharmacological treatment is administered for adverse events, the medications used should be recorded in the combined therapy record.

➤ **End Date and Time**

The completion date for AE must be entered in accordance with the filling requirements specified in the CRF and SAE report forms.

➤ **Outcomes**

The outcome of adverse events must be classified as one of the following:

- Recovery/Cure: The "End date of (severe) adverse event" should be specified.
- Improvement/Relief: The condition is recovering, improving, or stable.
- Not resolved/Not healed/Continuing: The event is ongoing.
- Recovery/ recovery with sequelae: This applies only when the subject experiences persistent or lifelong sequelae, such as blindness due to diabetes or hemiplegia following a stroke. The "end date of (severe) adverse event" should be specified.
- Death: The time of death must be recorded when a adverse event results in mortality.
- Unknown: Researchers were unable to determine adverse events, such as loss to follow-up of subjects.

1.1.6. Follow-up of adverse events

Following the initial AE report, investigators are required to proactively follow up with each subject and provide the sponsor with additional information regarding the subject's condition. The frequency of follow-ups should be determined based on the severity of the adverse event, standard

clinical practice, and trial protocol requirements. Any adverse events not resolved at this visit should be re-assessed and documented during the next visit; concomitant medications should be collected and recorded; for cases treated at a local hospital, medical records and medication information from that facility should be obtained whenever possible. Specific requirements must comply with the relevant Standard Operating Procedures (SOPs) of the healthcare institution.

For adverse events (AEs) that have not fully resolved or stabilized by the end of the safety follow-up period (regardless of causality), follow-up must continue until recovery/health restoration (returning to baseline levels or full recovery), with or without sequelae, clinical stability, a reasonable explanation provided, subject death, or loss to follow-up.

1.2. Serious Adverse Events (SAEs)

1.2.1. Definition of Serious Adverse Events

Serious Adverse Events (SAEs) refer to death, life-threatening conditions, permanent or severe disability or loss of function, hospitalization or prolonged hospital stay, congenital abnormalities or birth defects, as well as other significant medical events occurring in subjects after administration of the investigational drug.

Table 1 Definition of Serious Adverse Events

SAE definition	Guiding Principles
die	AE was the direct or primary cause of the subject's death.
threat to life	The occurrence of adverse events poses an immediate risk of death to the subject. This does not include adverse events that may lead to death if they progress severely, such as drug-induced hepatitis without subsequent hepatic failure.
Permanent or severe disability or loss of function	Any adverse event that causes injury, impairment, or disruption to the subject's physiological functions, anatomical structures, or both, as well as to physical activity or quality of life.
Hospitalization or extension of hospital stay is required	Events requiring extended treatment in hospitalized subjects should be recorded as SAEs. Examples of such events include transfer from routine hospital care to the intensive care unit (ICU), or cases where the event results in an extension of the planned hospital stay. The following hospitalization conditions should not be considered SAEs: - To be observed in the emergency department or another hospital ward for no more than 24 hours without resulting in hospitalization (unless it is considered a significant medical event or life-threatening); - An elective surgery that had been planned prior to signing the informed consent

	form; • Hospitalization is required for routine health examinations (e.g., conventional colonoscopy). • Hospitalization not intended for disease treatment, which was planned prior to enrollment, and must be documented; • Admission is unrelated to health conditions and does not require medical/surgical intervention (e.g., due to family reasons).
Congenital abnormalities or birth defects	The offspring of the subjects exhibit congenital abnormalities and malformations.
Other significant medical events	Medical and scientific judgment must be employed to determine whether reporting should be expedited for other conditions. For instance, significant medical events may not immediately pose a threat to life, death, or hospitalization; however, when medical interventions are required to prevent any of these outcomes, they are generally considered severe. Examples include critical treatments in the emergency room, allergic bronchospasm occurring at home, non-hospitalized cachexia or convulsions, as well as drug dependence or addiction.

1.2.2. Reporting of severe adverse events

For any serious adverse event occurring during clinical trials, regardless of its association with the investigational drug, investigators must provide immediate emergency treatment and complete the Serious Adverse Event (SAE) Report Form provided by the sponsor within 24 hours of notification, signing it and indicating the date before submitting it to the sponsor and/or the CRO designated by the sponsor. The SAE report must include detailed records of symptom description, severity level, occurrence time, intervention timing, measures taken, follow-up duration and method, as well as outcome information. For reports involving fatal events, investigators shall provide additional required documentation to the sponsor and the ethics committee, such as autopsy reports and final medical reports.

Researchers must provide their assessment of causality when reporting serious adverse events. If the researcher's causality assessment is missing or unavailable, the sponsor or a CRO designated by the sponsor (as stipulated in the project contract) shall immediately contact the researcher and submit an urgent medical inquiry requesting this assessment. Should the researcher be temporarily unable to provide the causality assessment, the sponsor shall make a judgment until the assessment can ultimately be obtained from the researcher.

If investigators cannot determine whether a adverse event constitutes a serious adverse event, it

shall be classified as such until its nature is established.

Upon receiving the relevant safety information for the clinical trial provided by the sponsor, investigators shall promptly acknowledge receipt and review it, consider whether to make corresponding adjustments to the subject's treatment regimen, communicate with the subject as early as possible when necessary, and report any suspected or unexpected serious adverse reactions provided by the sponsor to the ethics committee.

1.2.3. Follow-up of severe adverse events

The follow-up requirements for SAEs are identical to those for AEs. Investigators must promptly complete the Serious Adverse Event (SAE) Report Form and submit it to the sponsor and the sponsor-designated CRO in accordance with the SAE reporting protocol (as stipulated in the project contract).

1.2.4. Suspected unexpected serious adverse reaction

The following content is formulated in accordance with the "Standards and Procedures for Rapid Reporting of Safety Data During Drug Clinical Trials" issued by the CDE.

1.2.5. Definition of suspected unexpected serious adverse reactions

A Suspected Unexpected Serious Adverse Reaction (SUSAR) refers to a serious adverse reaction whose clinical manifestations in terms of nature and severity exceed those described in existing documentation such as the Investigator's Brochure, package insert of marketed drugs, or product characteristics summary. An unexpected adverse reaction is defined as one whose nature, severity, consequences, or frequency differs from the expected risks outlined in the current relevant information (e.g., the Investigator's Brochure). The Investigator's Brochure serves as the primary document providing reference criteria for determining whether an adverse reaction is expected or unexpected. Examples include: (1) Acute renal failure listed as an adverse reaction in the Investigator's Brochure but occurring during the trial as interstitial nephritis, which should be classified as an unexpected adverse reaction; (2) Pneumonia listed as an adverse reaction in the Investigator's Brochure but presenting as acute severe pneumonia during the trial, also qualifying as an unexpected adverse reaction.

1.2.6. Reports of suspected unexpected serious adverse reactions

Upon receiving any safety-related information from any source, the sponsor shall immediately analyze and evaluate it, including its severity, relevance to the investigational drug, and whether it constitutes an expected event. The sponsor shall promptly report SUSARs to all investigators participating in the clinical trial, the clinical trial site, the ethics committee, as well as the drug regulatory authorities and health authorities.

(1) The start/end dates for rapid reporting are as follows: the start date is the clinical trial approval date or the implied approval date set by the national drug regulatory authority, and the end date is the completion date of the last follow-up visit with a domestic participant (unintended serious adverse reactions occurring between the conclusion of clinical trial follow-up and the receipt of the review approval decision must also be reported via rapid reporting).

(2) Timeframe for the rapid report

- Lethal or life-threatening SUSAR cases: Report the initial occurrence within 7 days, and provide updated follow-up information within the subsequent 8 days.
- Non-fatal or non-life-threatening SUSARs: Report the first occurrence within 15 days.
- The day when the sponsor or CRO (as stipulated in the project contract) first becomes aware of the valid signed report is designated as Day 0.

1.2.7. Follow-up for the rapid report

After submitting the initial report (SUSAR), sponsors shall continue to monitor serious adverse reactions and promptly report any new information or modifications to previous reports in the form of follow-up reports, with a reporting deadline of 15 days from the date of obtaining new information.

1.3. Reports on other potential serious safety risk information

(1) Other potential serious safety risk information should also be reported by the sponsor to the National Medical Products Administration (NMPA) as soon as possible, with medical and scientific assessments conducted for each specific scenario.

(2) Generally, information that significantly impacts the risk-benefit assessment of a drug, may necessitate consideration of changes in drug usage, or affects the overall drug development process falls into this category.

I. For known severe adverse reactions, an increased incidence rate indicates clinical significance;

I. For known severe adverse reactions, an increased incidence rate indicates clinical significance;

II. Poses significant harm to exposed populations, such as when the drug is ineffective in treating life-threatening diseases;

III. Significant safety findings (e.g., carcinogenicity) from recently completed animal studies.

IV. Latest global safety warnings for similar drugs, or newly identified safety concerns disclosed in recently completed epidemiological studies or academic conference papers.

1.4. Adverse Events of Special Concern (ASEI)

Specifically monitored adverse events (AESIs) refer to those that require close monitoring to enhance the understanding of the safety profile of the investigational drug. The specifically monitored adverse events in this study include:

- hypersensitivity ;
- Cytokine Release Syndrome;

1.5. encyesis

Male and female subjects of reproductive age must consent to adopting effective, medically approved contraceptive measures from the initiation of first medication administration until 6 months after discontinuation. Investigators should emphasize the duration requirements for contraceptive use during informed consent and advise subjects regarding potential fetal effects of the investigational drug.

During the study period, female subjects must immediately discontinue the investigational drug and notify the investigator upon becoming pregnant. Such subjects are required to withdraw from the study. The investigator shall complete the Pregnancy Report Form within the same timeframe as severe adverse event reporting and submit it to the sponsor and the sponsor-designated CRO within 24 hours, while monitoring the pregnancy outcome (e.g., pregnancy termination or delivery) until three months after pregnancy conclusion or neonatal birth. For male subjects, if their sexual partner becomes pregnant during treatment, this must also be reported immediately to the investigator. The investigator will obtain a separate informed consent form from the female partner to collect pregnancy-related information and will follow up with her until three months after pregnancy conclusion or delivery.

Pregnancy itself does not constitute a SAE; however, abnormal pregnancy outcomes (such as spontaneous abortion, induced abortion for medical reasons, congenital anomalies, malformations, or fetal/neonatal death) must be recorded and reported as SAEs when completing the pregnancy report. The subject should receive appropriate monitoring and care until the completion of the pregnancy.

2. Statistical Considerations

The detailed summary and statistical analysis methods for the data collected in this study will be included in the Statistical Analysis Plan (SAP), which will be developed after the protocol and CRFs are finalized and finalized before database locking and data unblinding. The SAP will provide detailed specifications and descriptions for all statistical analyses planned, based on the key characteristics of the protocol. Any modifications to this protocol that the sponsor or principal investigator determines to significantly impact the statistical analysis plan must result in a revision of the SAP to align with the

2.1. Statistical Hypothesis

2.1.1. Sample Size Estimation

This study is a multicenter Phase II clinical trial, with Phase IIa serving as the dose exploration phase where safety and tolerability at doses of 0.5 mg/kg, 1 mg/kg, and other doses will be sequentially evaluated. Each dose group is planned to enroll 12 subjects; the sample size for the dose exploration phase does not require calculation based on statistical assumptions.

Phase IIb is a randomized, double-blind, placebo-controlled trial. The appropriate dose and primary efficacy endpoint will be determined based on the results of Phase IIa, without estimating the required sample size based on statistical assumptions. For efficacy exploration purposes, the provisional total sample size is set at 72 subjects (36 in the AS1501 group and 36 in the placebo group).

2.1.2. Analysis Population

Phase IIa analysis population

Subjects will be analyzed using the following analytical datasets:

- Safety Analysis Set: All subjects who received at least one dose (or any portion thereof) of the study drug (AS1501) and had recorded safety parameters. This analysis set will be used for all safety analyses, with subjects analyzed based on their actual treatment regimen.
- Full Analysis Set: All subjects who received at least one dose of the study drug (AS1501). This analysis set will be used for all efficacy analyses.
- Pharmacokinetic analysis cohort: All subjects who received at least one dose of AS1501, completed blood sampling at $\geq 60\%$ (3/5) of the first consecutive dosing points, did not experience protocol deviations that significantly affected PK parameters (e.g., C_{max}, AUC), and had at least one evaluable PK parameter. This cohort will be used for all pharmacokinetic analyses, with subjects analyzed based on their actual treatment regimen.
- Pharmacokinetic analysis cohort: All subjects who received at least one dose of the study drug and had at least one post-treatment pharmacokinetic (biomarker) measurement. Subjects will be analyzed based on their treatment regimen.
- Immunogenicity Analysis Cohort: All subjects who received at least one dose of the study drug and had at least one post-treatment immunogenicity measurement. This cohort will be used for immunogenicity analysis, with subjects analyzed based on their actual treatment

regimen.

Phase IIb analysis population

Subjects will be analyzed using the following analytical datasets:

- Safety Analysis Set: All subjects who received at least one dose (or any portion thereof) of the study drug (AS1501 or placebo) and had recorded safety parameters. This analysis set will be used for all safety analyses, with subjects analyzed based on their actual treatment regimen.
- Full Analysis Set: All randomized subjects who received at least one dose of the study drug (AS1501 or placebo). This analysis set will be used for all efficacy analyses, and subjects will be analyzed according to their assigned treatment group.
- Eligible analysis cohort: All randomized subjects who met the inclusion criteria, did not meet the exclusion criteria, completed at least 3 doses (75%) of the study drug (AS1501), and experienced no major protocol deviations affecting efficacy evaluation. This analysis cohort will be used to supplement the efficacy analysis based on FAS.
- Pharmacokinetic analysis cohort: All subjects who received at least one dose of the study drug (AS1501) and had at least one evaluable plasma concentration measurement. This cohort will be used for all pharmacokinetic analyses, with subjects analyzed based on their actual treatment regimen.
- Pharmacokinetic analysis cohort: All randomized subjects who received at least one dose of the study drug (AS1501 or placebo) and had at least one post-treatment pharmacokinetic (biomarker) measurement. Subjects will be analyzed according to their assigned treatment group.
- Immunoorigidity analysis cohort: All subjects who received at least one dose of the study drug (AS1501 or placebo) and had at least one post-treatment immunoorigidity measurement. This cohort will be used for immunoorigidity analysis, with subjects analyzed according to their randomized treatment assignment.

2.2. statistical analysis

2.2.1. conventional method

Statistical descriptions for categorical variables are presented as case counts and percentages (%); those for continuous variables include case counts, missing values, mean, standard deviation, median, lower quartile, upper quartile, minimum, and maximum. Unless otherwise specified, hypothesis tests

will be conducted as two-sided tests at a significance level of 0.05, with parameter confidence intervals estimated using the 95% confidence interval.

2.2.2. Subject Distribution

Summarize the enrollment, medication administration, dropout, exclusion, and dataset partitioning details for each group of subjects, accompanied by a flowchart illustrating subject distribution.

The list describes the details of subject dropout exclusions.

2.2.3. Demography and Baseline Characteristics

Statistical descriptions were provided for the baseline characteristics of each group, including demographic data and laboratory test results. The baseline descriptive statistics of the study protocol were discussed, specifying whether statistical inference was performed.

2.2.4. Pharmacokinetic and Pharmacodynamic Analysis

Pharmacokinetic Evaluation

Blood drug concentrations in subjects will be summarized by dose group and sampling time point. Pharmacokinetic parameters will be calculated using a non-ventricular model and summarized by dose group using descriptive statistics. For peak time (T_{max}), statistical measures include the number of valid observations, median, minimum, and maximum values; for other parameters, statistical measures include the number of valid observations, mean, standard deviation, coefficient of variation, median, minimum, and maximum values. Pharmacokinetic analysis will be performed based on the pharmacokinetic analysis dataset and the actual treatment regimen administered to subjects.

PD Parameter Evaluation

Pharmacokinetic (biomarker) measurements at each visit and their changes from baseline will be summarized by descriptive statistics by dose group. Pharmacokinetic analysis will be performed based on the pharmacokinetic analysis dataset.

Group PK/PD Evaluation

Based on data characteristics and the drug's mechanism of action, select an appropriate model for population PK/PD or PPK studies to identify covariates influencing PK/PD.

2.2.5. safety analysis

Adverse events and adverse reactions:

Analysis of adverse events and adverse reactions during medication administration: summarize the number of cases, occurrences, and percentages of adverse events, adverse reactions, trial discontinuation

due to adverse events, deaths attributable to adverse events, and severe adverse events across all study groups; perform intergroup comparisons of incidence rates when necessary.

According to the MedDRA dictionary, adverse events and adverse reactions were classified using Systemic Organ Classification (SOC) and Preferred Term (PT) codes, and the number of occurrences, frequency, and percentages of adverse events and adverse reactions were statistically analyzed based on SOC/PT classifications.

Classify adverse events and adverse reactions by SOC/PT and severity level to calculate the number of occurrences, instances, and percentages. For each category of adverse event in a subject, it is counted only once under the highest severity level within the same term (SOC or PT).

List all adverse events (including those occurring during non-pharmacological periods), adverse reactions, serious adverse events, study discontinuations due to adverse events, deaths attributable to adverse events, and specially monitored adverse events separately.

laboratory examination :

The statistical time points for each laboratory test parameter include baseline, all post Baseline visitation time points, and the final visitation time point. Measurements at each time point, as well as the minimum and maximum values of post-Baseline measurements, the observed values at the final visitation, and their relative changes from baseline, are calculated separately by dose group and overall cohort.

Using a clinical judgment cross-tabulation before and after medication to compare the normal and abnormal changes of various indicators.

Provide a detailed list of laboratory test parameters by group.

vital sign :

The statistical time points for vital sign measurements include baseline, post-baseline visitation time points, and early withdrawal visitation time points. Descriptive statistics were calculated separately by group and overall for baseline values, post-baseline visitation values, and study completion data. Normality and abnormality of each parameter before and after medication administration were compared using a clinical judgment cross-tabulation (where applicable).

Physical examination and electrocardiogram (ECG):

Physical examination items: general condition, head, neck, lymph nodes, chest, abdomen, musculoskeletal system, skin, nervous system, and other areas. Normal and abnormal changes before and after medication in each dose group were compared using a clinical judgment cross-tabulation table.

Electrocardiogram (ECG) parameters include: heart rate, PR interval, QRS complex, and QTc interval. Statistical analysis was performed for baseline values, values at each post-baseline visit time point, minimum and maximum values of post-baseline measurements, as well as the observed values at the final visit and their changes from baseline, stratified by dose group and overall. Normality and abnormality changes before and after medication were compared across all dose groups using a clinical judgment cross-tabulation.

Provide a detailed list of physical examinations and electrocardiogram (ECG) tests categorized by group.

2.2.6. efficiency analysis

Effectiveness variables will be analyzed per visit. For continuous variables, measurement values and their changes from baseline will be summarized by dose group using descriptive statistics; categorical variables will be summarized by dose group using frequency and percentage. Variations in continuous variable measurements relative to baseline will be compared between dose groups using covariance analysis. If data do not follow a normal distribution assumption, the Wilcoxon rank-sum test will be employed. For binary variables, the Fisher's exact test will be used to compare differences between dose groups.

The efficacy analysis is primarily based on the full analysis set and the randomized treatment assignments of subjects. The protocol-compliant analysis set will be utilized for supplementary analysis.

3. Data Processing and Record Storage

All participating institutions shall comply with Good Clinical Practice (GCP) and ICH GCP guidelines, as well as applicable laws and regulations regarding subject privacy protection, and maintain all medical and research records required for the study.

3.1. Responsibilities for Data Collection and Management

Prior to initiating clinical trial data management, the Data Management Department shall develop a Data Management Plan (DMP) tailored to the specific project requirements. The DMP is a dynamic document prepared by data managers in accordance with the clinical trial protocol, which comprehensively defines and documents all data management tasks for a particular clinical trial, including personnel roles, responsibilities, and operational protocols.

3.2. Data Collection and Methods

This study employed an Electronic Data Capture System (EDC) for data collection. Data administrators/database programmers created separate accounts with tailored access permissions based

on their roles to operate the EDC system.

The data recorded in the Electronic Case Report Form (eCRF) must originate from the source documents and remain consistent with the source data. All source documents shall be kept clear and organized to ensure accurate data identification. Permanent copies of study visit records shall serve as source documents for documenting enrolled subjects' data. Data entry personnel shall promptly and accurately transfer information from source documents such as study medical records into the eCRF.

3.3. Data Cleaning and Question Resolution

Data cleaning involves a series of steps including data verification (systematic and manual logical checks), identification of anomalies, responses from investigators/research assistants, data updates, and resolution of identified issues.

Data administrators and monitors regularly perform data cleansing via the EDC system, while medical monitors conduct periodic medical reviews through the same system. For any EDC-related inquiries, investigators or research assistants provide online responses and/or correct erroneous data. The original inquirer confirms the resolved data and may re-submit inquiries if necessary.

3.4. Data Modification and Review

Data entry personnel or investigators may modify the data after verification; any modifications must be documented with a justification on the eCRF. Investigators have full authority to review all final data.

3.5. Data Locking and Export

After all data has been reviewed and confirmed as accurate, the data administrator locks the data. Any modifications made after locking require signature confirmation from the principal investigator, study investigators, and the data administrator before implementation. The final data is imported by the data administrator into the designated database for statistical analysis by statisticians.

Upon completion of the study, the eCRF will be printed and archived as needed. The Data Management Center will store the electronic data.

Data management tasks not explicitly specified in the protocol shall be implemented in accordance with the data management plan of this trial.

3.6. Storage of research records

To ensure evaluation and oversight by the National Medical Products Administration and the sponsor, investigators shall retain all study materials in compliance with Good Clinical Practice (GCP), including all source data records of participants such as clinical trial records or original record books, signed informed consent forms, and detailed documentation of drug distribution. Upon completion of

the trial, PDF versions of electronic case reports (eCRFs) for all participants from each research center shall be burned onto CDs and stored at the respective centers. Study materials must be retained for at least 5 years after the termination of the clinical trial or 5 years following approval of the investigational drug for marketing; no materials may be destroyed without prior written consent from the sponsor.

All data from this clinical study are owned by the sponsor. Without the written consent of the sponsor, investigators may not provide such data to any third party in any form, except as required by the National Medical Products Administration (NMPA).

3.7. Research Publication and Data Sharing Policies

All data and results obtained from this trial, as well as the intellectual property rights associated with them, shall belong to the sponsor. Investigators may utilize the trial data for scientific purposes, provided that prior consultation with the sponsor is required before publication, and any intended publication content must obtain written consent from the sponsor.

The sponsor acknowledges that the investigator has the right to publish the results upon completion of the trial. However, the investigator must submit the full article or abstract to the sponsor prior to submission; the sponsor will review the manuscript for accuracy (to ensure consistency with regulatory submissions), verify that no confidential or patent information is disclosed, and supplement any necessary details as appropriate. In case of disagreement between the sponsor and the investigator, both parties will discuss the proposed publication content to reach a mutually satisfactory resolution.

For multicenter studies, the initial publication must be based on data from all centers and analyzed by a biostatistician designated or approved by the sponsor in accordance with the protocol, rather than being performed by individual investigators. Investigators participating in multicenter studies may not publish data collected from a single center or a limited number of centers prior to the initial publication of data from all centers, unless formal consent has been obtained from all other investigators and the sponsor.

The authors of the manuscript are determined through mutual agreement among all parties involved. For submissions containing aggregated data, authors may include members from each participating center as well as staff members of the sponsor.

The publication of the trial results will be detailed in the clinical research protocol.

4. Ethical Guidelines and Informed Consent

4.1. Ethical Ethics

The conduct of this trial complies with Good Clinical Practice (GCP) guidelines, the Helsinki Declaration, relevant regulations, and the review opinions of the ethics committee. Investigators must

ensure that the trial has been reviewed and approved by a qualified ethics committee that meets GCP requirements. During the review process, both the sponsor and investigators must submit the trial protocol, informed consent forms, and other necessary documents to the ethics committee. The sponsor may only provide the investigational drug upon receiving official approval from the ethics committee. Additionally, the ethics committee must be notified of any subsequent protocol amendments that may affect participant safety or continued participation, as well as any serious adverse events occurring during the trial. Investigators are responsible for reporting trial progress to the ethics committee and must promptly submit all communications with the ethics committee to the sponsor. When reviewing and approving the trial protocol, the ethics committee must confirm the protocol title, protocol number, and specify the reviewed documents along with the review date. Throughout the trial, any additions or revisions to the trial protocol or informed consent forms must obtain written approval from the relevant regulatory authorities in accordance with regulations.

4.2. informed consent

Before participating in this trial, subjects must provide informed consent to ensure the protection of their legal rights and interests. The investigator is responsible for providing subjects or their designated representatives with a comprehensive and detailed explanation of the study objectives, methodology, drug effects, expected benefits, potential adverse reactions, and associated risks. Subjects should be fully informed of their rights, risks, and potential benefits, and any new information regarding the investigational drug should be communicated promptly. Subjects must be assured that participation in this clinical trial is entirely voluntary, and they may withdraw from the trial at any time without penalty. The investigator and sponsor reserve the right to access, retain, and statistically analyze the subjects' data in accordance with applicable regulations. The informed consent form must specify the version number, date of preparation, or revision date, and must be signed by both the subject (and their legal guardian) with a dated signature. The investigator involved in obtaining consent must also sign the form, and an independent witness capable of verifying the subject's agreement may additionally sign the document. One copy of the informed consent form shall be retained by each party. If significant new information regarding the investigational drug emerges, the consent form must be amended in writing, approved by the ethics committee, and the subject's consent re-obtained.

4.3. confidentiality and privacy

The confidentiality and privacy rights of participants are strictly safeguarded by the participating investigators, their staff, the sponsor, and all associated intervention measures. This confidentiality extends beyond clinical information related to participants to include tests involving biological sample

analysis and genetic testing. Consequently, the study protocol, documentation, data, and all other information generated during the study shall remain strictly confidential. No research materials or data may be disclosed to unauthorized third parties without the written consent of the sponsor.

All research activities will be conducted in a private setting whenever possible.

Study monitors, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), regulatory authorities, or representatives of the pharmaceutical company supplying the study product may review all documents and records that need to be retained by investigators, including but not limited to medical records of study subjects (from offices, clinics, or hospitals) and pharmacy records. Clinical research centers should permit access to these records.

The subjects' contact information will be securely stored at each clinical research center for internal use during the study. Upon study completion, all records will remain stored in a secure location for a period exceeding that required by IRB review, institutional regulations, or sponsor specifications.

5. Quality Assurance and Quality Control

All parties involved in clinical trials, including sponsors, research centers, and CROs, shall implement appropriate quality control measures to ensure that the trials comply with the Helsinki Declaration, Good Clinical Practice (GCP), relevant laws and regulations, and standard operating procedures (SOPs).

5.1. Requirements for Applicants

- 1) The sponsor or its representative must provide the latest version of the Investigator's Brochure (IB) for each unregistered clinical trial compound. For marketed products, provide the product package insert and/or the most up-to-date product information.
- 2) The sponsor or its representative, together with the investigators and statistical experts, develops the final version of the clinical trial protocol. Upon reaching consensus among all parties, the protocol is signed and submitted to the ethics committee.
- 3) The sponsor or its representative shall provide all investigators with an adequate number of case report forms and instruct them on how to fill them out and store them properly.
- 4) The sponsor or its representative shall provide all investigators with sufficient trial materials to support and enable them to conduct the trials in accordance with the established protocol.
- 5) The sponsor or its representative reserves the right to terminate the clinical trial prematurely due to recurrent protocol violations or other well-founded ethical concerns. In such cases, both parties shall conduct review and consultation to implement necessary measures while ensuring patient rights.

5.2. Duties of the monitor appointed by the sponsor or its representative

Clinical trial monitors serve as the primary liaison between sponsors and investigators. They must adhere to Good Clinical Practice (GCP) and Standard Operating Procedures (SOPs), conducting regular or ad hoc visits to research sites to perform clinical monitoring, oversee trial progress, verify the accuracy and completeness of all data records and reports, ensure eCRF entries are correct and consistent with original data, and guarantee compliance with the trial protocol. Investigators are required to provide full cooperation with monitor activities. Key responsibilities of monitors include:

- 1) Prior to the trial, confirm that the participating institution meets all required conditions, including adequate personnel allocation and training, complete and properly functioning laboratory equipment, availability of all necessary testing facilities, an estimated sufficient number of subjects, and ensure that all trial participants are familiar with the requirements specified in the trial protocol.
- 2) During the trial, monitor the investigator's adherence to the protocol; confirm that informed consent has been obtained from all participants prior to enrollment; assess the participant inclusion rate and trial progress; and verify that enrolled participants meet the eligibility criteria.
- 3) Confirm that all data records and reports are accurate and complete, with all case report forms correctly filled out and consistent with the original data. All errors or omissions have been corrected or noted, signed by the investigator and dated accordingly. Any dose adjustments, treatment changes, concomitant medications, comorbidities, loss to follow-up, or missed examinations for each subject must be confirmed and documented.
- 4) Ensure that all adverse events are properly documented, with severe adverse events reported and recorded within the specified timeframe; verify that the investigational drug is supplied, stored, distributed, and recalled in compliance with relevant regulations, with corresponding records maintained.
- 5) Assist investigators with necessary notifications and application procedures, and report trial data and results to the sponsor.
- 6) All visits that the investigator failed to conduct, trials that were not performed, examinations that were not carried out, as well as whether errors or omissions have been corrected, shall be recorded clearly and accurately.
- 7) After each visit, a written monitoring report shall be completed, specifying the monitoring date and time, the name of the monitor, and any findings identified during the monitoring.

5.3. Requirements for researchers

- 1) Researchers participating in clinical trials must possess specialized expertise, qualifications, and capabilities in clinical trials. Following qualification review, the personnel roster should remain relatively stable.
- 2) Prior to patient enrollment, investigators should explain the purpose of the clinical study to the participant or their family members, obtain informed consent, and have them sign the informed consent form.
- 3) Personally execute and supervise the conduct of clinical studies;
- 4) Fill out the case report form (eCRF) carefully in accordance with the requirements;
- 5) In conjunction with regular visits by clinical monitors appointed by the sponsor or their representatives;
- 6) Completely retain records of laboratory tests, clinical records, and the patient's original medical documentation;
- 7) In the event of a serious adverse event, investigators must identify its cause and take appropriate measures, while reporting it to the principal investigator.
- 8) Follow-up of severe adverse events.

5.4. Inspection and Examination

As required by the trial, when the sponsor or a third-party entity conducts an audit, or when the drug regulatory authority inspects the study, direct access to the original trial documents should be permitted; data in the eCRF must derive directly from the original data.

5.5. Plan Deviation

All requirements specified in the study protocol must be strictly adhered to. Any intentional or unintentional deviation from or violation of the trial protocol and Good Clinical Practice (GCP) principles shall be classified as a protocol deviation. During monitoring, if a protocol deviation is identified, the investigator or monitor shall complete a protocol deviation record detailing the detection time, event occurrence time and process, cause, and corresponding corrective actions; this record shall be signed by the investigator and communicated to both the ethics committee and the sponsor. The statistical analysis report and summary report shall analyze and report the impact of any protocol deviations on the final data and conclusions.

An evaluation shall be conducted when significant protocol deviations occur. If necessary, the sponsor may terminate this study prematurely.

5.6. Safety Supervision

The Safety Monitoring Committee will consist of representatives from the sponsor and principal investigators. This committee will review all data, provide a detailed explanation of dose-limiting toxicity based on the protocol, and determine whether further dose escalation or reduction is warranted. The Data Review Committee (DRC) will examine all safety data; any treatment-related deaths will also trigger a DRC review to ensure subject safety. The DRC shall implement the charter provisions established and approved at the committee's inception, which shall clearly define all data elements requiring DRC evaluation and the responsibilities of each party involved.

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7. Appendix

Appendix 1 Cockcroft-Gault formula

The unit of creatinine is mg/dl:

$$\text{Male Ccr (ml/min)} = \frac{(140 - \text{age}) \times (\text{weight})}{72 \times \text{serum creatinine}}$$

$$\text{Female Ccr (ml/min)} = \frac{0.85 \times (140 - \text{age}) \times (\text{weight})}{72 \times \text{serum creatinine}}$$

$$\frac{0.85 \times (140 - \text{age}) \times (\text{weight})}{72 \times \text{serum creatinine}}$$

The unit of creatinine is μmol/L:

$$\text{Male Ccr (ml/min)} = \frac{(140 - \text{age}) \times (\text{weight})}{0.818 \times \text{serum creatinine}}$$

$$\text{Female Ccr (ml/min)} = \frac{0.85 \times (140 - \text{age}) \times (\text{weight})}{0.818 \times \text{serum creatinine}}$$

$$\frac{0.85 \times (140 - \text{age}) \times (\text{weight})}{0.818 \times \text{serum creatinine}}$$

Note: Age is expressed in years, and weight is expressed in kilograms (kg).

Appendix 12: Information on the central laboratory and the sample destruction company

Central Laboratory 1: Pharmacokinetics, Immunogenicity Testing, Serum TRAIL Expression

Name: Junhe Zhengyuan (Shanghai) Biomedical Technology Co., Ltd.

Unified Social Credit Code: 91310115MA7CFRF017

Address: 1st Floor, Building 2, No.88 Chenhui Road, China (Shanghai) Pilot Free Trade Zone

Central Laboratory 2: TRAIL Expression in NK Cells

Name: Guangzhou Jinmanli Medical Technology Co., Ltd.

Unified Social Credit Code: 91440101MA9ULJB15Y

Address: Units 601–609,6th Floor, Building (2), No.5 Helix 4 Road, International Bio Island, Huangpu
District, Guangzhou

Sample Destruction Company 1

Name: Shanghai Solid Waste Disposal Co., Ltd.

Unified Social Credit Code: 913101147294906145

Address: No.2491 Jiazhu Highway, Jiading District, Shanghai

Sample Destruction Company Two

Name: Guangzhou HuanTou Renewable Resources Utilization Co., Ltd.

Unified Social Credit Code: 91440112MA9YE1YP4T

Address: No.128 Tandong Road, Fushan Village, Xinlong Town, Huangpu District, Guangzhou City

Appendix 3: Child-Pugh scoring scale

The Child-Pugh classification system is a clinically widely used grading standard for quantitatively assessing hepatic reserve function in patients with cirrhosis. First proposed by Child in 1964, this system categorizes five patient parameters — including general condition, ascites, serum bilirubin, serum albumin concentration, and prothrombin time — into three levels assigned scores of 1, 2, or 3 respectively. The total score ranges from 1 to 15 points, with the highest possible score indicating optimal liver reserve function and the lowest representing severe impairment (higher scores correspond to poorer liver reserve function). However, due to challenges in evaluating patients' general condition, Child subsequently introduced the inclusion of hepatic encephalopathy presence and severity as alternative indicators, leading to the current widely adopted modified Child-Pugh classification system.

The specific classification criteria are shown in the table below:

Clinical Assessment Criteria	1 point	2 points	3 points
Hepatic encephalopathy (stage)	not have	1 - 2	3 - 4
ascites	not have	mild	Moderate to severe
Total bilirubin (umol/L)	<34	34 - 51	>51
albumin (g/L)	>35	28-35	<28
Prothrombin time prolonged (seconds)	<4	4-6	>6

Grade A: 5–6 points – The surgical risk is low with the best prognosis, and the survival rate ranges from 100% to 85% within 1–2 years.

Grade B: 7–9 points – moderate surgical risk; survival rate of 80%–60% at 1–2 years.

Class C: ≥ 10 points indicates a high surgical risk and the poorest prognosis, with a 1–2 year survival rate of 45%–35%.

Appendix 4: AARC Scoring Scale

In 2017, the APASL study enrolled 1,402 patients with acute liver failure (ACLF) meeting the APASL criteria. The study identified bilirubin levels, hepatic encephalopathy severity scores, international normalized ratio (INR), serum lactate concentration, and serum creatinine as prognostic factors, and developed the ACLF-AARC scoring system. The AARC score is calculated as the sum of five parameters, ranging from a minimum of 5 to a maximum of 15 points, divided into three grades: Grade I (5–7 points), Grade II (8–10 points), and Grade III (11–15 points). The specific scoring criteria for each parameter are shown in the table below:

fraction	Total Bilirubin (mg/dL)	HE by stages	INR	Blood lactate (mmol/L)	serum creatinine (mg/dL)
1	<15	0	<1.8	<1.5	<0.7
2	15~25	I~II	1.8~2.5	1.5~2.5	0.7~1.5
3	>25	III~IV	>2.5	>2.5	>1.5

Appendix 5 MELD score for end-stage liver disease models

The End-Stage Liver Disease Model (MELD) is a system primarily utilizing serum bilirubin, international normalized ratio of prothrombin time, and serum creatinine levels to assess end-stage liver disease. As the application of the MELD score in predicting mortality from end-stage liver disease and liver transplantation outcomes has become increasingly established, its scope has expanded to include severe hepatitis and hepatocellular carcinoma.

In 2000, Malinchoc et al. first applied the MELD model to predict mortality in patients with end-stage liver disease undergoing transjugular intrahepatic portosystemic shunt surgery and demonstrated that MELD could accurately predict both mortality rates and postoperative survival times in these patients. The calculation formula was as follows: $R = 0.957 \times \ln(\text{creatinine mg/dL}) + 0.378 \times \ln(\text{bilirubin mg/dL}) + 1.120 \times \ln(\text{INR}) + 0.643 \times (\text{where the value is 0 for causes of cirrhosis such as cholestatic or alcoholic cirrhosis, and 1 for other causes})$. A higher R-value indicates greater risk and lower survival rates.

For computational convenience, Kamath et al. refined the formula to $R = 3.8 \times \ln[\text{bilirubin (mg/dL)}] + 11.2 \times \ln(\text{INR}) + 9.6 \times \ln[\text{creatinine (mg/dL)}] + 6.4 \times (\text{where } 0 \text{ indicates biliary or alcoholic liver disease, and } 1 \text{ indicates other causes}).$

Note: INR is referred to as the International Normalized Ratio in Chinese. It is calculated based on prothrombin time (PT) and the International Sensitivity Index (ISI) of the testing reagent, enabling comparability of PT measurements across different laboratories and reagents while facilitating standardized medication practices. The formula is $\text{INR} = (\text{PT}_{\text{test}} / \text{PT}_{\text{normal}}) \times \text{ISI}$, where ISI is denoted by an superscript.

Appendix 6: Diagnostic Criteria for SIRS

A diagnosis of SIRS can be made if two or more of the following clinical manifestations are present:	
temperature	<36°C or>38°C
heart rate	>90 times per minute
breathing rate	>20 times per minute or PCO ₂ <32 mmHg (1 mmHg = 0.133 kPa)
leucocyte count	<4.0×10 ⁹ /L or>12×10 ⁹ /L

Appendix 7 CRS Grading Criteria (ASTCT Consensus)

CRS Symptom 1	Level 1	Level 2	Level 3	Level 4
Fever 2	Body temperature ≥38°C	Body temperature ≥38°C	Body temperature ≥38°C	Body temperature ≥ 38 °C
		merge		
Hypotension	not have	No hypertensive medications are required.	A vasopressor agent is required; vasopressin may or may not be necessary.	Multiple vasopressor agents are required (excluding vasopressin).
		And/or 3		

hyoxemia	not have	requires low-flow nasal cannula oxygen therapy (4) or mechanical ventilation	Oxygen therapy is required using a high-flow nasal cannula, facial mask, non-rebreathing mask, or Venturi mask.	Positive pressure oxygen therapy is required (e.g., CPAP, BiPAP, endotracheal intubation, or mechanical ventilation).
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Note: CPAP: Continuous Positive Airway Pressure; BiPAP: Dual-Level Positive Airway Pressure.

- 1 Organ toxicity associated with CRS can be graded according to the CTCAE V5.0 criteria, but this does not affect the CRS grading.
- 2 Fever is defined as a body temperature $\geq 38^{\circ}\text{C}$ not caused by other etiologies. After administration of antipyretics or corticosteroids, fever is no longer required to assess the severity of subsequent CRS. In such cases, CRS classification is determined by hypotension and/or hypoxemia.
- 3 The CRS classification is determined by more severe events: hypotension or hypoxia requires exclusion of other causes. For example, a patient with a body temperature of 39.5°C requiring one antihypertensive agent for hypotension and low-flow nasal cannula oxygen therapy for hypoxia is classified as Grade 3 CRS.
- 4 Low-flow nasal cannula oxygen therapy is defined as an oxygen flow rate $\leq 6\text{ L/min}$. Low-flow oxygen therapy also includes mechanical ventilation, which is sometimes employed in pediatric patients. High-flow nasal cannula oxygen therapy is defined as an oxygen flow rate $>6\text{ L/min}$.