Investigation of Therapeutic Effects and Neural Mechanism of Bumetanide on Preschool-aged Children with Autism Spectrum Disorder: A Randomized, Double-blind Placebo-controlled Trial

CLINICAL TRIAL PROCEDURE

(TRANSLATED VERSION)

STUDY DESIGN

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SUMMARY

Research: Investigation of the therapeutic effects and neural mechanism of bumetanide on preschool-aged children with autism spectrum disorder: a randomized, double-blind, placebo-controlled trial

Primary objective: To investigate the efficacy and safety of low-dose bumetanide (1 mg/day) in preschool-aged children with autism spectrum disorder in China

Secondary objective: To investigate the treatment mechanism of bumetanide using neuroimaging and computational neurobiology approaches

Enrolled cases: 120 cases

Study design: The study is a randomised, double-blind, placebo-controlled, parallel-group design

Study population: Children with ASD aged 3 to 6 years old

Intervention: The intervention group will receive a safe dose (1 mg/day) of bumetanide orally for 3 months; the control group will receive a placebo orally for 3 months. Bumetanide and placebo tablets will be identical in appearance, smell, and taste.

Observatory outcomes: The primary end point is the change from baseline to 3-month follow-up in Childhood Autism Rating Scale (CARS). The secondary end points include the score of the Clinical Global Impressions-Improvement (CGI-I), and the changes from baseline to 3-month follow-up in Autism Diagnosis Observation Scale (ADOS), Social Response Scale (SRS), Repetitive Behaviour Scale-Revised (RBS-R), Symbolic Game Scale (SPT), Short Sensory Profile Report (SSPR) and Chinese Communicative Development Inventory (CDDI). The exploratory end points are the changes in neuroimaging MRI/MRS scans (structure and neurotransmitter concentrations), EEG recordings, and metabolic parameters before and after treatment, as well as SNP association analysis.

Safety evaluation: Patients will be monitored at 1 week and 1 month after the initiation of treatment and at the end of the treatment period. For safety evaluation, the blood potassium concentration, blood uric acid concentration, increased urine elimination and other indicators will be regularly monitored and used for safety evaluation.

Sample size: the total number of cases for this study is 120, which will be 60 in the bumetanide group and 60 in the placebo group.

Inclusion criteria: **A.** Between 3 to 6 years of age; **B**. meet the diagnostic criteria for the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-5); **C**. meet the diagnostic criteria for the Autism Diagnostic Observation Scale (ADOS) and/or Autism Diagnostic Interview Scale (Revised Edition) (ADI-R); **D**. Childhood Autism Rating Scale (CARS) total score of no less than 30; **E**. no access to any behavioural intervention; **F**. signed written informed consent.

Exclusion criteria: **A**. Liver or kidney dysfunction; **B**. a history of allergy to sulfa drugs; **C**. abnormal

electrocardiogram; **D**. genetic and/or chromosomal abnormalities; **E**. neurological and psychiatric diseases (such as epilepsy, schizophrenia, and so on); **F**. currently using melatonin for the treatment of sleep disorders or cessation of such treatment for less than 3 weeks; **G**. Exclusion criteria for magnetic resonance scanning, in addition to the above criteria, including any contraindications of MRI scanning and any previous reports of traumatic brain injury.

Study medication: Patients in the treatment group will receive a safe oral dose (1 mg/day) of bumetanide (0.5 mg/dose; twice daily in the morning/afternoon with an interval of 8 hours is recommended, such as 08:00 AM and 16:00 PM; it is not recommended to take the medication at night) for 3 months with close follow-up monitoring. Patients in the control group will receive an oral placebo twice daily for 3 months, with close follow-up monitoring. To make sure these enrolled patients take the study medication following the clinician's instruction, follow-up phone calls for a medication compliance check will be made in the first week and the first month after initiation of treatment. At the end of treatment, the patient will be instructed to return the remaining medication.

Study time: From May 2017 to October 2019

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ASD	**Autism Spectrum Disorder**
DSM-5	**Diagnostic and Statistical Manual of Mental Disorders, 5th Edition**
CARS	**Childhood Autism Rating Scale**
ADOS	**Autism Diagnosis Observation Scale**
ADI-R	**Autism Diagnostic Interview-Revised**
IQ	**Intelligence Quotient**
DQ	**Developmental Quotient**
CGI	**Clinical Global Impressions Scale**
CGI-I	**Clinical Global Impressions Scale - Improvement**
SPT	**Symbolic Play Test**
SRS	**Social Responsiveness Scale**
RBS-R	**Repetitive Behaviour Scale-Revised**
SSPR	**Short Sensory Profile Report**
CCDI	**Chinese Communicative Development Inventory**
MRI	**Magnetic Resonance Imaging**
MRS	**Magnetic Resonance Spectroscopy**
GABA	**γ-aminobutyric acid**
FDA	**The Food and Drug Administration**
AE	**Adverse Event**
SAE	**Severe Adverse Event**
CRF	**Case Reporting Form**
EEG	**Electroencephalogram**
GWAS	**Genome-Wide Association Study**

Investigation of Therapeutic Effects and Neural Mechanism of Bumetanide on Preschool-aged Children with Autism Spectrum Disorder: A Randomized, Double-blind Placebo-controlled Trial

1. Research Background

Childhood autism (also named Autism Spectrum Disorder, ASD) was first reported and named by American psychiatrist Kanner in 1943. It is a severe neurodevelopment disorder characterized by impairments in social interaction and language communication, and by repetitive stereotypes and narrowed interests. It is more common in males and occurs more frequently in early childhood. The incidence of ASD continues to increase worldwide and in China. As of March 2012, the US Centers for Disease Control and Prevention reported a prevalence 1/88 for ASD [1]. Approximately 60% to 70% of children with ASD exhibit varying degrees of mental retardation and 38% of ASD patients cannot live independently and require life-long care, resulting in a heavy burden on families and society [2].

In recent years, it is believed that personalized medicine is the main strategy in the treatment of neuropsychiatric diseases including ASD. Traditional symptom-based diagnosis and treatment of diseases is gradually moving towards an etiology-based diagnosis and treatment in practice. Taking ASD as an example, in DSM-5 issued in 2013, ASD was defined as a neurodevelopmental disorder which is initiated in early life. Current evidence supports the idea that the development of ASD is largely influenced by factors associated with genes, environment, and gene-environment interactions. In addition, children with ASD have strong heterogeneity in behavioural symptoms and severity phenotypes due to strong plasticity in early brain development, further increasing the difficulty of ASD diagnosis and treatment. Therefore, the development of personalized intervention while considering clinical symptoms, genetic background, risk factors, and others has been emphasized to target different symptoms in ASD [3].

To date, behavioural interventions remain the first-line choice for ASD; these include Applied Behavioural Analysis (ABA), Denver's Early Intervention Model, etc. However, these interventions were slow to be established in China and the development of intervention training and related education programs have not yet been perfected. The number of qualified behavioural intervention facilities is limited in many regions; this variation in intervention quality makes the therapeutic effects worrying. The bottleneck in the development of behavioural interventions in China poses further limitations in children with ASD and their families after a clear diagnosis has been made. This results in them either searching for a reliable intervention program, or giving up on appropriate treatment, thus postponing the timing before young children with ASD receive treatment. Therefore, the development of medicinal drug options and clinical applications for ASD treatment is considered a breakthrough.

However, worldwide, there are few evidence-based and effective medicinal treatments for ASD; in particular there is no approved medication that targets the core symptoms. Moreover, to date, there is no medication for treating ASD core symptoms in China. In the US, the FDA have approved two schizophrenia drugs (Risperidone in 2006 and Aripiprazole in 2009) for treating several symptoms for

patients with ASD. These two drugs are considered to relieve symptoms related to irritability provoking emotion, self-harm, and aggressive behaviour, but have no significant effects on core symptoms and social communication. Research suggests that there is a different pathogenesis in autism compared to schizophrenia, and ASD is associated with an imbalanced inhibitory transmission in the brain (such as insufficient inhibition of GABAergic neurons) [4]. The above mechanism has been shown in an animal model of ASD, and also in ASD patients through the new magnetic resonance mass spectrometry in 2015 [5-7]. It is believed that developing a drug that targets inhibitory neurotransmission signaling could be the cure for ASD.

Bumetanide, a classic diuretic widely used in clinical practice, has recently been shown to act on GABAergic signaling by enhancing the inhibitory function of GABAergic signaling in the central nervous system [8]. Responses to treatment in animal models have been good [9]. Bumetanide has been prescribed for adults since 1975 and for children since 1986 to treat hypertension, bronchopulmonary dysplasia, and nephritis syndrome. More recently, small pilot studies have shown that a 3- to 9-month treatment of bumetanide could improve core social symptoms in some autistic children, such as face recognition, paresthesia, and social perception. Moreover, the side effects of bumetanide are significantly less than those of Risperidone and Aripiprazole. The dose of 1 mg/day is within a safe range for nephrology, and the side effects are minimal for young children (3 years old) [10-13]. Therefore, bumetanide is now considered a novel and interesting medicinal drug for the treatment of ASD. However, the mechanism of bumetanide in the human autistic brain (where it targets and how it functions) remains largely unknown.

Based on the current status of ASD treatment in China, this project aims to investigate the efficacy and safety of bumetanide treatment in autistic children aged 3 to 6 years and to study the treatment mechanism. We will conduct a randomized, double-blind, placebo-controlled trial coupled with state-of-the-art magnetic resonance spectrometry (MRS) to evaluate alterations in GABA and other neurotransmitters in specific brain regions before and after treatment, and to identify a potential surrogate biomarker for therapeutic evaluation.

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2. Research objectives

ASD is an early-onset neurodevelopmental disorder with an urgent need for effective medication options that attenuate the core symptoms. This randomised, double-blind, placebo-controlled trial of bumetanide is mainly for the following purposes:

(1) To determine the safety and efficacy of low-dose bumetanide (1 mg/day) in preschool-aged children with ASD in China;

(2) To explore the treatment mechanism in human autistic brain using neuroimaging and computational neurobiology approaches.

3. Research design

This is a 3-month randomised, double-blind, placebo-controlled trial to investigate the therapeutic effects and neural mechanism of bumetanide on preschool-aged children with ASD. A total of 120 cases of children with ASD aged 3-6 years will be enrolled. We will recruit eligible children from outpatient clinics at the Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Those who meet any of the inclusion criteria and no exclusion criteria will be enrolled and randomly assigned to either of two treatment groups of 60 patients each.

During the 3-month treatment period, the patients in the treatment group will receive a safe dose of 1 mg/day of bumetanide with close follow-up monitoring; the children in the control group will receive a placebo with close follow-up monitoring. Bumetanide and placebo tablets will be identical in

appearance, smell, and taste. At baseline, the behaviour evaluation and history record for each patient will be completed. The Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview-Revised (ADI-R) will be assessed by two clinician psychologists who have been formally trained by a research reliable rater and have acquired certificates. The intelligence quotients (IQ), or developmental quotients (DQ) will be assessed by an experienced assessor using the Wechsler Intelligence Scale for Children-Revised (WISC-R, Chinese version) for patients ⩾ 6 years old and the Wechsler Preschool and Primary Scale of Intelligence (WPPSI, Chinese version) for patients < 6 years old but ⩾ 4, and the Gesell Developmental Schedules Chinese version for patients < 48 months old. The Childhood Autism Rating Scale (CARS) will be evaluated by an experienced developmental behavioural pediatrician. The Symbolic Play Test (SPT) will be assessed by experienced clinicians. The Social Responsiveness Scale (SRS), Repetitive Behaviour Scale-Revised (RBS-R), Short Sensory Profile Report (SSPR), Chinese Communicative Development Inventory (CDDI), etc. will be completed by the patient's caregiver. Biological samples (i.e., blood) will be collected and appropriately stored for future studies. One week and 1 month after the initiation of treatment, follow-up phone calls will be scheduled to monitor/record any potential medicine-related adverse events (AE) and medication-compliance. The clinical re-evaluation of CARS, ADOS, SPT will be performed after 3 months of treatment. The Clinical Global Impressions Scale-Improvement (CGI-I) will be assessed by an experienced clinician after the treatment period. The SRS, RBS-R, SSPR, CCDI, etc. will be again completed by the patient's caregiver after 3 months of treatment. Biological samples will be collected and appropriately stored for future studies. Before and after treatment MRI scans (including structural scans and MRS measuring GABA and glutamate) will be collected. In addition, body weight, height, blood potassium concentration, blood uric acid concentration, increased urine elimination, and other indicators of each patient will be regularly monitored during the treatment period.

4. Research subject

4.1 Indication

The indication for this study is ASD.

4.2 Sample size and calculation method

This study anticipates enrolling 120 autistic patients aged 3-6 in one research center.

Calculation of sample size: see **12.3**

4.3 Inclusion criteria

A. between 3 to 6 years old;
B. meet the diagnostic criteria for the DSM-5;
C. meet the diagnostic criteria for the ADOS and/or ADI-R;
D. CARS total score of no less than 30;
E. no access to any behavioural intervention;
F. signed written informed consent

4.4 Exclusion criteria

A. with liver or kidney dysfunction;
B. a history of allergy to sulfa drugs;
C. with abnormal electrocardiogram;
D. with genetic and chromosomal abnormalities;
E. with neurological diseases (such as epilepsy, schizophrenia, etc.);
F. currently using melatonin for the treatment of sleep disorders or cessation of such treatment for less than 3 weeks;
G. Exclusion criteria for magnetic resonance scanning, in addition to the above criteria, including any contraindications of MRI scanning and any previous reports of traumatic brain injury,

4.5 Subject withdrawal and discontinuation criteria

Subjects can decide to leave the study at any time for any reason without any consequences. The investigator can decide to withdraw a subject for reasons including, but not limited to, obvious hypokalemia, hyperuricemia, severe physical illness after enrollment, ineligibility for inclusion criteria or exclusion criteria after enrollment, or lost visit. Patients will not be replaced after withdrawal from the study. No standard follow-up as described in this protocol will be performed on patients who are withdrawn from treatment, although these patients will be asked to participate in the study visit at 3 months (at least for the primary endpoint of the study).

5. Study medication

5.1 Bumetanide

① Drug name and specification: Bumetanide tablets (1mg/tablet)
② Dosage: 0.5 tablet to be taken orally, twice daily, in the morning and afternoon, interval 8 hours, i.e., 08:00 AM and 16:00 PM; it is not recommended to take the medication at night.
③ Treatment course: The whole treatment course is 3 months.
④ Preparation: The bumetanide tablets (The Approval Number of The National Drug Administration: H45020938) will be supplied by Guilin Pharmaceutical CO., LTD located on 43 Qilidian Road, Guilin, Guangxi Province, China).

5.2 Placebo

① Drug name and specification: placebo (contains pharmaceutical excipients such as starch, but does not contain pharmaceutical ingredients)
② Dosage: 0.5 tablet to be taken orally, twice daily, in the morning and afternoon, interval 8 hours, i.e., 08:00 AM and 16:00 PM; it is not recommended to take the medication at night.
③ Treatment course: The whole treatment course is 3 months.
④ Preparation: The placebo tablets will be supplied by the Pharmaceutical Department of Shanghai Xiangshan Hospital of Traditional Chinese Medicine located on 528 Middle Fuxing Road, Shanghai, China. The Department currently holds a Manufacturing Certificate # 20160005HZ issued by Shanghai Provincial FDA on January 1, 2016.

The placebo production will be in compliance with GMP quality standard and the manufacturing operations. The placebo tablets look identical to the bumetanide tablets, although the placebo tablet will not contain any active ingredient. Both active and placebo tablets will be manufactured using the same tablet manufacturing process, to be packaged into HDPE bottles and labelled respectively. Quality

control procedures, to be conducted by the manufacturer, will be used to ensure that by weight, appearance, and other features, such as disintegration time, friability and microbial limits, the bumetanide and placebo tablets are identical. Tablets will be only released to the Xinhua Hospital if they conform to the quality control specifications.

5.3 Others

To prevent side effects such as hypokalemia and hyperuricemia, the patients will be advised to eat plenty of potassium-rich fruits and drink more water during the treatment. At the same time, follow-up phone calls for side effects and medication compliance will be made after the first week and the first month of treatment. At the end of the treatment, patients will be instructed to return the remaining medication. During the study period, if the treatment is interrupted due to intolerance of medication or other adverse events, the situation will be documented and discussed with physician immediately to decide whether or not to discontinue the treatment. The interruption of the trial medication should generally not exceed 7 days and the total dosage should not be <90%. If the treatment is discontinued by a physician who is not involved in the study due to an AE, or if the patient decides to withdraw on his/her own volition, the situation must be reported to the Ethical Committee immediately; relevant information should be collected and recorded. Whether or not to resume the interrupted treatment should be discussed with a physician and the principle investigator when the medical condition of the patient has improved.

6. Follow-up plan

6.1 Follow-up schedule

❶ The number of follow-up visits for the subjects was set at 4 times,

- Follow-up 1 (V1): baseline
- Follow-up 2 (V2): week 1
- Follow-up 3 (V3): week 4
- Follow-up 4 (V4): week 12

❷ Unscheduled follow-up (USV): unlimited

6.2 Follow-up content: Scheduled plan

Follow-up time / Content	V0	V1	V2	V3	V4
	-1m	Day0	Day7	Day30	Day90
Informed consent	*				
Inclusion criteria	*				
Exclusion criteria	*				
Randomisation		*			
Medical history collection	*	*			*
Physical examination	*				*
DQ/IQ	*				

ADIR	*				
ADOS	*				*
CARS	*				*
CGI-I					*
SRS	*				*
SSPR	*				*
SPT	*				*
CCDI	*				*
RBS-R	*				*
MRI	*				*
EEG	*				*
Biological samples collection	*				*
Blood potassium	*		*	*	*
Blood uric acid	*		*	*	*
AE			*	*	*
SAE			*	*	*
Medication compliance			*	*	*

7. Study parameters/endpoints

7.1 Primary study parameter/endpoint

The CARS is made up of 15 subscales. Each of the subscales is scored on a continuum from normal to severely abnormal. A score of 1 indicates that the child's behaviour is within normal limits for his or her age, 2 is scored for mildly abnormal, 3 for moderately abnormal, and 4 for severely abnormal behaviour. The three midpoints between adjacent ratings are used when the child's behaviour appears to be between any two of the four integer values. The child's age must be considered in making each rating. Individuals with scores of <30 are not considered to be autistic. Two criteria are used that best distinguish two groups of autistic children. The designation of severe autism is used for those children whose total score exceeds 36 and who have a rating of 3 or higher on five or more of the 15 subscales. All scores that do not result in classification into these two extreme categories will be placed in the middle category of mild to moderate psychosis. At both baseline and outcome time points, the CARS will be administered by a clinician (developmental-behavioural pediatrician), clinically involved in the field of ASD. The clinician will be independent from this study.

7.2 Secondary study parameters/endpoints

❶The Clinical Global Impressions-Improvement scale (CGI-I) is part of CGI, which is a three-item scale used to assess treatment response in psychiatric patients. (1) The Global Improvement item requires the clinician to rate how much the patient's illness has improved or worsened relative to a baseline state. Compared to condition at baseline, a patient's illness is compared to change over time, and rated according to the patient's condition: 1=very much improved since the initiation of treatment;

2=much improved; 3=minimally improved; 4=no change from baseline (the initiation of treatment); 5=minimally worse; 6=much worse; 7=very much worse since the initiation of treatment." (2) The CGI-I will be evaluated by an experienced clinician.

❷The ADOS, designed as a diagnostic tool for ASD, is a semi-structured, standardized observation instrument, including domains of reciprocal social interaction, communication, and repetitive and stereotyped behaviours. All domains with higher scores indicate greater severity. One of 3 modules and 1 of 5 scoring algorithms will be chosen, depending on language abilities and age. The same module across time points for each subject will be applied to ensure consistency. The ADOS will be assessed by a clinician psychologist.

❸At both baseline and follow-up, the SPT will be administered by an experienced clinician.

❹At both baseline and follow-up, the SRS, RBS-R, SSPR, CCDI, etc. will be completed by the patient's caregiver.

Note:

1) The assessors will be independent from the study.
2) Assessor-rated outcomes including the ADOS, CARS, etc. will be administered by the same assessors before and after the treatment period.
3) During the initiation stage of the study, all detailed information including the research protocol, the enrolment procedure and the content of pre-treatment education classes will be delivered to the assessors. The assessors will not participate in the group allocation of subjects and are not responsible for drug administration.
4) From the preliminary study, it has shown that polyuria is most likely to occur within 3 hours of taking bumetanide. Therefore, in order to minimize the impact of urination on assessor blindness, the subjects will be evaluated about 4 hours after taking bumetanide. In addition, each subject will be asked to urinate before evaluations take place. For younger subjects, the caregiver will be asked to change the child into a new diaper before evaluation.

7.3 Exploratory study parameters/endpoints

Neuroimaging parameters: MRS will be used to measure GABA and glutamate neurotransmitter concentrations in the insular cortex before and after treatment (Appendix-study protocol).

Electroencephalogram (EEG): Before and after treatment, the EEG signals will be collected and recorded using 128 channel EGI Electroencephalographic system (Appendix-study protocol).

Genetic factors: Biological samples will be collected before and after treatment (Appendix-study protocol). It will be decided later whether to perform genetic tests based on the funding situation.

Metabolomics factors: Biological samples will be collected before and after treatment. It will be decided later whether to perform metabolism tests based on the funding situation.

8. Safety evaluation parameters

8.1 Laboratory inspection parameters

Biochemical examination: blood potassium concentration, blood uric acid concentration

8.2 Symptoms and physical signs

Medical history (preexisting medical condition) and other information will be collected at the baseline. Physical examinations including weight, height, etc. will be conducted at the baseline and post-treatment visits. Other symptoms will be rated and recorded according to the Rating Scale for Side-effects at the baseline and post-treatment, as well as in follow-up phone call interviews.

8.3 Adverse events and their incidence See **9**

9. Adverse events and severe adverse events

9.1 Adverse events

An adverse event (AE) refers to any symptom, sign, or clinical manifestation that is incompatible with the purpose of treatment after the trial has started, even if it is not related to treatment. Any AE must be recorded and followed up.

9.2 Record of adverse events

All AEs that occur after signing the informed consent will be recorded. For the purposes of this study, AE is defined as any abnormal and unexpected signs, symptoms, or transient illnesses associated with clinical research. The content of each AE record should include the following:

❶ Duration of adverse event: Start time and end time；

❷ Severity of adverse event: Grade 4, Mild-does not affect daily activities
Grade 3, Moderate-affects daily activities
Grade 2, Severe-incapacity
Grade 1, Life threatening；

❸ The relationship between adverse events and study medications: the investigators will determine the correlation between adverse events and study medications as follows: definitely related / likely related / possibly related / possibly irrelevant / irrelevant；

❹ Treatment of adverse events: the adverse events will be flowed and monitored by investigators until the symptoms disappear or stabilize; if the adverse events persist at the end of the study, they should be followed up again within one month after the end of the study.

The study medication, bumetanide, will be used off-label in this study. Although it will be used at a low dose, the potential risks are the risks of side effects of the medication, including anorexia, fatigue, hypokalemia, and hyperuricemia. Among them, anorexia and fatigue are common phenomena during the first month of medication, which can be relieved by ingesting potassium-rich fruits or other dietary supplements. Hypokalemia, hyperuricemia and other side effects are relatively rare, less than 1% reported in the literature, which occurred in a small number of cases in our previous experiments. We will examine and monitor the blood potassium, uric acid for patients at 1-week, 1-month and the end of treatment period. Oral potassium supplementation will treat hypokalemia and followed by a close monitoring. Hyperuricemia will be adjusted by supplementation of more drinking water and referred to a doctor specialist if necessary.

9.3 Serious adverse events

A serious adverse event refers to any untoward medical occurrence or effect that at any dose:

❶ results in death;

❷ is life threatening (at the time of the event);

❸ requires hospitalization;

❹ results in persistent or significant disability or incapacity;

Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the patient or may require an intervention to prevent one of the outcomes listed above.

The investigator will decide whether the SAE is correlated to the use of the study medication or not, and whether the patient has to (temporarily) discontinue the medication treatment. The patient will continue to be followed and monitored according to the study guidelines, unless either the patient or the investigator decides to prematurely stop participation in the trial, in which case an early-discontinuation visit will be planned.

All SAEs that occur any time after the inclusion of the patient in the trial (at the time when the patient or the legal guardian signs the informed consent) up to 30 days after the patient completes or discontinues the study, must be reported. Completion is defined as either the completion of the last visit or contact (e.g. phone contact with the investigator or designee), or the time after the last dose of the study medication, whichever is later. Discontinuation is defined as the date a subject and/or investigator decides that the subject no longer fulfils the requirements for any further study visit or evaluation. The SAEs, whether or not considered drug-related or expected, must be reported within 24 hours (at the earliest possibility) by the physician/investigator to the Medical Administration in Xinhua Hospital. All SAE reports will be presented the Ethical Committee and/or reported in accordance with local laws and regulations.

10. Ethical committee and informed consent

10.1 Ethical committee

Prior to the initiation of this trial, the research protocol and informed consent will have been reviewed and approved by the ethics committees of Xinhua Hospital. During the research, any changes to the research plan should be filed with the ethics committee in a timely manner. When SAEs occur, the ethics committee will be notified within 24 hours.

10.2 Informed consent

Before enrollment, the investigator needs to explain in detail to each legal applicant (or legal representative) with ASD the nature, purpose, procedures, duration, the benefits, and the possible risks of this clinical study. Only after the informed consent form signed by the legal representative of the subject can they enter the study.

11. Randomisation, blinding, treatment allocation and unblinding

11.1 Randomisation and blinding

This is a randomised, double-blind, placebo-controlled trial. Patients who have given informed consent and who are eligible for all inclusion criteria and do not have any of the exclusion criteria, will be randomly assigned to one of two study groups. Patients will be randomized by use of a block randomisation scheme. The list with randomisation numbers will be generated by appropriate personnel in an external consultancy with an SAS algorithm. The personnel in charge of randomisation will retain a complete set of the randomisation scheme and will not participate in other aspects of the study to ensures that blindness is properly maintained throughout the trial. The sealed envelopes with random numbers and bumetanide/placebo bottles will be prepared by the external consultancy. After receiving the study medication (bumetanide or placebo) from Guilin Pharmaceutical CO., LTD and the Pharmaceutical Department of Shanghai Xiangshan Hospital, respectively, the external consultancy will package the study medication into numbered HDPE bottles according to the randomisation numbers, and then ship the bottles with study medication to a pharmacist in the study team. The study medication will be securely stored by the pharmacist at room temperature. The sealed envelopes with randomisation numbers will be properly maintained by a nurse in the study team. Patients, caregivers, and the whole study team will be masked to treatment assignment.

11.2 Treatment allocation

Patients are randomly assigned in a 1:1 ratio to receive bumetanide or placebo according to the order of enrollment. The study medication (bumetanide or placebo tablets) in numbered bottles will be provided by the pharmacist according to the prescription and associated randomisation number.

11.3 Unblinding

Before subjects receive treatment, the personnel in charge of randomisation will retain a complete set of randomisation numbers. In the event that subjects are prematurely discontinued from the trial, it will be necessary to avoid breaking the blind whenever possible, in order to protect the integrity of the study. If an emergency necessitates that the blind be broken, only the personnel in charge of randomisation will have access to the unblinding codes and will be given the names of the staff with authority to request that the blind be broken (Dr. Fei Li). The personnel in charge of randomisation can be reached 24 hours a day by cellphone to rapidly access subject unblinding codes. Any intentional or unintentional breaking of the blind should be reported and explained at the end of the trial, irrespective of the reason for its occurrence. The procedure and timing for revealing the treatment assignments should be documented and the inspector will be notified immediately.

12. Data management and statistical analysis

12.1 Data collection

The principle investigator and assisting investigators will fill in the data of each subject in the CRF on time according to the requirements of the research. The principle investigator will keep the subject's original data (copy) as much as possible to ensure accuracy.

12.2 Data Safety Monitoring Board

An independent Data Safety Monitoring Board (DSMB) will regularly review subject safety data

and evaluate the efficacy of the study intervention throughout the duration of the trial, and provide recommendations to the principle investigator to either continue, amend, or terminate the study based on the information above.

12.3 Evidence of sample size determination

This study anticipates enrolling 120 autistic patients aged 3-6 years in one research center.

Calculation of sample size:

❶ Because the placebo is selected in the control group, the comparison type of the clinical efficacy of this clinical trial was a superiority study;

❷ Based on the historical clinical studies in patients with ASD, it is assumed that the reduction of the CARS score after a 3-month treatment of bumetanide is in average ($\mu_A - \mu_B = 2.00$ points, $\sigma = 3.10$) more than that in the placebo group. Assuming an enrollment ratio of 1:1 between the bumetanide group and the treatment group, the sample size was calculated at a 2-sided significance level $\alpha = 0.05$ to have a statistical power of $1-\beta = 90\%$ using the formula for calculating the sample size of the statistical superiority test $n_A = n_B = 2\sigma^2(Z_{1-\alpha/2} + Z_{1-\beta})^2 / (\mu_A - \mu_B)^2$. The sample size is calculated by PASS software and at least 51 cases for each group will be required.

❸ Considering a potential dropout rate at 15%, the total number of cases for this study is 120, which will be 60 in the bumetanide group and 60 in the placebo group.

12.4 Selection of statistical analysis data

Intention-to-treat (ITT) analysis is a method for analyzing results in a prospective randomized study where all subjects who are randomized are included in the statistical analysis and analyzed according to the group they were originally assigned. In the statistical analysis, the last observation data will be used to analyze the efficacy and adverse reactions intentionally, when there is missing data for any subject. If a patient withdraws from the trial before taking any dose of the drug, then a modified ITT is going to be employed by excluding this patient.

12.5 Statistical analysis of the population

❶ Analysis of primary study parameter/endpoint: analysis using the ITT population;

❷ Analysis of secondary study parameters/endpoints: analysis using the ITT population;

❸ Analysis of exploratory study parameters/endpoints: analysis using the population with data collected;

❹ Safety analysis: analysis using the population with at least one dose of study medication used.

12.6 Statistical analysis method

Data will be pre-processed and/or analyzed using SAS9.2, LCModel, and R (https://www.r-project.org/).

❶ Analysis of primary parameter/endpoint:

The treatment effect of bumetanide is assessed by the change of the total score of CARS from baseline to 3-month using a mixed-effect model. In this model, we assumed individualized random

intercepts, and tested the treatment effect by the interaction term, treatment (0, placebo; 1, bumetanide) × time (0, baseline before treatment; 1, month 3 after treatment). Due the double-blind randomisation, the bumetanide group and the placebo group are supposed to be well balanced in terms of sex, age, symptom severity (ADOS, ADIR, CARS), and intellectual assessment (DQ or IQ). Therefore, these covariates are not included in this mixed-effect model. In case that these covariates are not balanced between these two groups before the treatment, these baseline covariates need to be included in this mixed-effect model. The normality of the model residuals was assessed with the Shapiro–Wilk normality test, and homogeneity of variance across groups was evaluated with Levene's test. If at least one of the two tests were significant, a permutation-based mixed-effects model was established by 3000 random permutations of the group label using the *permlmer* function in R package "*predictmeans*" v.1.0.1.

If the treatment effect is significant in the CARS total score, the same mixed-effect model is applied to the candidate subscales, based on the potential findings from our randomised, open-labelled, clinical trial (Chinese Clinical Trial Registry at ChiCTR-OPC-16008336). Adjustment for multiple comparisons among the candidate subscales is performed at $p = 0.05$ using the false discovery rate approach (Benjamin-Hochberg adjusted-p value).

❷ Analysis of secondary parameter/endpoint:

The treatment effects of bumetanide on ADOS, SRS and others are assessed by the same statistical analyses as described for the primary parameter. CGI-I is used to confirm the treatment effect of bumetanide by a non-parametric inter-group comparison of CGI-I after treatment between these two groups, namely Kruskal-Wallis chi-square test.

❸ Analysis of exploratory parameter/endpoint:

The treatment effects of bumetanide on the neuroimaging is assessed by the same statistical analyses as described for the primary parameter. After the quality control for the neuroimaging data, patients with qualified neuroimaging data at both time points are entered into the mixed-effect model. The demographics and the baseline symptoms are compared between the patients with the neuroimaging data and the patients without the neuroimaging data to identify the possible selection bias in the neuroimaging data. The age, sex, and IQ are included in this model for the neuroimaging indicators.

Correlation coefficient between the change of symptom and the change of imaging indicators before and after treatment is calculated in each group. If the data is normal or approximately normal distribution, Pearson's correlation analysis is used; if the data does not meet the positive after conversion of variables Normal or close to normal distribution, using rank correlation analysis.

EEG and other indicators (i.e. metabolites in blood samples) are assessed by the statistical analyses as described in Appendix-study protocol.

❹ Safety analysis:

After enrollment, the changes of the test results of each vital sign compared to the baseline were described and counted, and the number of cases, mean, standard deviation, median, minimum, and maximum were calculated.

List all laboratory tests and descriptive statistics for the laboratory test indicators (based on the range of normal values and the investigator's judgment of clinical significance) and compare the bumetanide group and the control group with abnormal changes in laboratory test indicators after treatment and Clinically significant cases.

Summarize the occurrence of adverse events: i) Compare the number, incidence and incidence of adverse events in the bumetanide group and the control group, and list all adverse events, adverse events related to the study drug, and serious adverse events respectively in the two groups, important adverse events, and adverse events leading to shedding; ii) Compare the number, incidence and incidence of mild, moderate, and severe adverse events between the two groups; iii) List the number and number of adverse events.

❺ Analysis of withdrawal/discontinuation

The incidence of withdrawal/discontinuation in the two groups is analyzed. The reason of withdrawal/discontinuation such as adverse events, lack of efficacy, violation of the clinical protocol, or lost visit will be documented and analyzed. The incidence of withdrawal in two groups are compared using Fisher's exact probability method.

❻ Analysis of baseline characteristics

The comparison of baseline characteristics between these two groups includes: demography (age, sex and DQ/IQ status). Calculate the number of cases, mean, standard deviation, median, minimum, and maximum when the variable is a quantitative indicator; calculate the number of cases and composition ratio when the variable is a categorical indicator. When two groups of comparability tests are performed, the Welch's t test (t statistic, assuming non-equal variances) will be used for normal or close to normal distribution of measurement data, Wilcoxon rank sum test is used for skewed distributions, and chi-square test or Fisher exact probability method are used for count data.

13. Principal investigator and project leader

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