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The Inhibitory Effect of Anisodamine Hydrobromide Against Contraction of Intestinal Smooth Muscles Induced by Acetylcholine, With Its Pharmacokinetics and Mechanism Investigation

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Abstract

Objective: To investigate the inhibitory effects of Anisodamine hydrobromide (AniHBr) on acetylcholine (ACh)-induced contractions and pharmacodynamics, and the potential mechanism. **Methods:** The inhibitory effect of AniHBr on ACh-induced contraction of isolated rat and rabbit intestinal smooth muscles (n=10) was studied. The average strain was measured by a multiple physiological signal acquisition and processing system equipped with a muscle tension sensor. The plasma drug concentrations in Beagle dogs were detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to investigate the AniHbr pharmacokinetic profile. Based on network pharmacology, qRT-PCR and Western blot experiment used to detect the expression changes of key factors in IM-R069 cells. **Results:** Results showed that AniHBr significantly inhibited the Ach-induced contraction of rat and rabbit intestinal smooth muscles, reduced the average strain. A peak plasma concentration (C_{max}, 51.01±37.99 ng/mL) of AniHBr was observed within 0.72h post-dose. Moreover, the mean elimination half-life of AniHBr was 1.02 ± 0.19 h. Network pharmacology analysis identified 148 key targets of AniHBr associated with gastrointestinal diseases. qRT-PCR and Western blot experiment showed 20 ug/mL AniHBr significantly increased the mRNA expression of EFGR and STAT3, and suppressed PI3K-AKT signaling pathway. **Conclusions:** AniHBr effectively inhibits Ach-induced contraction of isolated rat and rabbit intestinal smooth muscles, might be through regulating EFGR, STAT3 and PI3K-AKT signaling pathway.

Keywords

anisodamine hydrobromide, bioavailability, pharmacodynamics, smooth muscle, PI3K-AKT signaling pathway

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1. Introduction

Abdominal cramping and pain are common symptoms in the general population, which occur due to functional and organic diseases.[1-5] The common etiologies for abdominal pain presenting to emergency ward are acute gastroenteritis, intestinal obstruction, irritable bowel syndrome and functional abdominal pain.[6-8] Antispasmodic medicines are widely used for the treatment of symptoms of abdominal pain or discomfort associated with cramping, either by prescription or as over-the-counter drugs.[9] In a variety of medicines, anticholinergics are agents that inhibit or block the actions of acetylcholine (ACh) on its parasympathetic nervous system receptors on smooth muscle cells of the gastrointestinal tract, thus relieving abdominal cramping and pain.[10-12]

Anisodamine is an active ingredient extracted from the root of a Chinese specialty plant *Scopolia tangutica* maxim in 1956.[13] It is a belladonna alkaloid, and its chemical structure is (2S) -3-hydroxy-2 phenyl-propionic acid (3S,6S) -6-hydroxy-8-methyl-8-azabicyclo [3.2.1] oct-3- ylester.[14,15] It has a long clinical history in China, reportedly first used as an analgesic by local people in the plateaus of Qinghai and Tibet.[13] Because of the limitations on natural source and extraction technique decades ago, natural anisodamine (anisodamine hydrobromide, AniHBr) cannot meet the huge clinical demands. Racemic anisodamine (RaceAni) as an alternative to AniHBr, has been marketed in China for many years since it was synthesized and characterized in 1975.[13]

Currently, with the development of the planting base and the production process of AniHBr, the production of AniHBr has been greatly improved.[16] It is necessary to pay more attention to the natural monomer AniHBr in the new era. Our previous studies have demonstrated that AniHBr could protect against lipopolysaccharide (LPS)-induced production of inflammatory cytokines, mitochondrial dysfunction, and oxidative stress, thereby alleviating the lipopolysaccharide-induced acute kidney injury.[17] AniHBr could also protect endothelial cells against LPS-induced increase in permeability and nitric oxide (NO) production *via* preserving the glycocalyx.[18] In addition, it has been reported that AniHBr may be a potential drug for treatment of the Coronavirus Disease 2019 by reducing inflammation and promoting microcirculation.[19] Thus, it is necessary to evaluate the efficacy and safety of AniHBr. In the present study, the inhibitory effects of AniHBr on ACh-induced contractions and pharmacodynamics were studied. Furthermore, since a variety of gastrointestinal disorders are associated with abdominal pain, we explored the potential targets of AniHBr using network pharmacology, and examined the expression changes of related factors through qRT-PCR and WB experiments.

2. Materials and Methods

2.1. Effects of AniHBr on Isolated Intestinal Smooth Muscles Induced by ACh

2.1.1. Animals

A total of forty Sprague-Dawley rats (half male and half female) weighing 180-220g were purchased from Chengdu Dashuo Biotechnology Co., Ltd., China, and housed in metabolic cages under standard conditions, with food and sterile water available ad libitum, in a room with a 12/12-h light/dark cycle and controlled temperature (20-25°C), moisture (40% -70%). A total of forty New Zealand rabbits (half male and half female) weighing 2.5-3kg were purchased from Pizhou Dongfang breeding Co., Ltd., China, and housed in a temperature-controlled (16-26 °C) room with a 12/12-h light/dark cycle. They were given standard food and tap water ad libitum. All experimental procedures involving animals were approved by the animal committee of Sichuan Industrial Institute of Antibiotics (No. SYXK 2019-021) and the ethics committee of Sichuan University (No. KS2021661). All animal experiments were conducted at Sichuan Industrial Institute of Antibiotic, China National Pharmaceutical Group Corp. Research period: September 2019 - March 2021.

2.1.2. Preparation of Isolated Intestinal Segments

Rats were euthanized by intraperitoneal injection of pentobarbital sodium[20] (50mg/kg; 201201, Beijing Chemical Reagent Co., Ltd, China) and rabbits were euthanized by intravenous injection pentobarbital sodium (30 mg/kg).[21] The abdominal cavity was cut along the middle line to avoid injuring the intestinal tract. About 2cm of duodenum was rapidly cut off, then the outer membrane fat and connective tissue were peeled off along the intestinal wall. The intestinal contents were rinsed three times with the Tyrode's solution containing (in mmol/L) 11.90 $NaHCO_3$ (P125162, Adamas, China), 136.89 NaCl (P1477664, GENERA-REAGENT, China), 2.68 KCl (P1492140, GENERA-REAGENT, China), 1.05 $MgSO_4 \cdot 7H_2O$ (P1043274, GENERA-REAGENT, China), 0.54 NaH_2PO_4 (P1425577, GENERA-REAGENT, China), 1.8 $CaCl_2$ (P1504983, GENERA-REAGENT, China) and 5.55 glucose (P1504976, GENERA-REAGENT, China). The isolated intestinal smooth muscles were suspended in an organ bath (SQG-4J, Chengdu Instrument Factory, China) containing 18 mL Tyrode's solution. Tyrode's solution was bubbled with a mixture of 95% oxygen and 5% carbon dioxide to adjust the pH to 7.4 at 37°C, as in previous works.[22] The isolated intestinal smooth muscles were allowed to equilibrate for 1 h with a preload

of 1 g.[23] During the equilibration time, the Tyrode's solution in organ bath was changed every 15 min. N represents the number of intestinal segments from n animals.

The reporting of this study conforms to ARRIVE 2.0 guidelines.[24]

2.1.3. Preparation Method of AniHBr

For drug preparations, 5 mg AniHBr tablets (Catalog No. 181001, 5mg/tablet, purity 99.3%, Chengdu First Pharmaceutical Co., Ltd, China) were fully ground, dissolved in 125 mL normal saline at concentration of 40 μg/mL as mother solution, and then added in the organ bath to reach the final drug concentrations.[17] The AniHBr tablets are now commercially available, with both the composition and purity assays conducted in accordance with the Chinese Pharmacopoeia (2020 edition).

2.1.4. AniHBr Treatment and Strain Measurement

When the intestinal smooth muscles were stable, ACh (final concentration 1×10^{-6}moL/L, P1226676, Adamas Reagent Co., Ltd, China) was added to precontract isolated intestinal smooth muscles.[25] After establishing a stable contraction of the intestinal segments, the isolated intestinal smooth muscles were treated with AniHBr. The final treatment concentrations of AniHBr for rats were 0, 0.5, 1.0, 2.0 μg/mL respectively, and for rabbits were 0, 0.25, 0.5, 1.0 μg/mL respectively. Multiple physiological signal acquisition and processing system (RM6240E, Chengdu Instrument Factory, China) equipped with a muscle tension sensor was used to measure and record the strain of the isolated intestinal smooth muscle. The average strain for each isolated intestinal smooth muscle was recorded three times and averaged respectively. The relative change in muscle strain was calculated as: (Effect value - Control value)/Control value × 100%. In which, the control value is the initial average strain prior to addition of ACh, and the effective value is the average strain induced by Ach or ACh+AniHBr.

2.2. Assessment of Pharmacokinetics

2.2.1. Animals

Six Beagle dogs (half male and half female) weighing 6.5~8.2 kg, were provided by the Beagle Breeding Center of Sichuan Musk Breeding Research Institute, China. All experimental procedures involving animals were approved by the animal committee of Sichuan Industrial Institute of Antibiotics (No. SYXK 2019-021) and the ethics committee of Sichuan University (No. KS2021661). All animal experiments were conducted at Sichuan Industrial Institute of Antibiotic, China National Pharmaceutical Group Corp.

2.2.2. AniHBr Dosing

After an overnight fast of 12 hours, the Beagle dogs received a single intragastric dose of 5 mg AniHBr according to the instructions. Food intake was allowed 4 hours after oral administration and water intake was permitted throughout the period.

2.2.3. Blood Sample Collection and Plasma Concentration Determination

For the oral pharmacokinetic study, blood from the vein of lower limbs was sampled in heparinized test tubes at 0 h (baseline), 0.17h, 0.33h, 0.5h, 1h, 2h, 3h, 4h, 5h, 6h, 6h, 8h, 10h, 12h, and 24h after drug administration.[26] After the blood collection, the plasma samples were separated immediately by centrifuged (H3-18KR, Hunan Kecheng Instrument Equipment Co., LTD, China) at 3000g for10 min. The collected plasma samples were stored at a -70°C ultra-low temperature freezer (DW-86L490, Haier Group, China) until analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[27] The LC-MS/MS analysis were performed on a Waters ACQUITY UPLC I-CLASS UPLC system (Waters Corporation, USA), coupled with ESI ion source, Xevo TQ-S detector, and UNIFI software (Waters Corporation, USA).

The LC conditions are as follows:

The LC conditions	
Column	BEH C18 (1.7 μm, 50 mm × 2.10 mm)
Mobile phase A	0.1% formic acid in water
Mobile phase B	Acetonitrile
Injection volume	1 μL
Column temperature	40 °C

The mobile phase gradient

Time (min)	Flow (mL/min)	A (%)	B (%)
0.0	0.5	90	10
0.6	0.5	0	100
1.0	0.5	0	100
1.5	0.5	90	10

2.2.4. Pharmacokinetic Analysis

A non-compartmental analysis (NCA) was applied (Phoenix WinNonlin version 7.0, Certara, USA) to determine: area under the concentration versus time curve (AUC) from time zero to T h ($AUC_{0\text{-}t}$), AUC from time zero to infinity ($AUC_{0\text{-}\infty}$), maximum concentration (C_{max}), time to C_{max} (T_{max}), rate constant λ_z), half-life ($T_{1/2(d)}$), apparent distribution volume (Vd/F), and clearance rate (Cl/F). AUC_{0-t} was calculated according to the linear trapezoidal rule.[26]

2.3. Network Pharmacology Research[28]

2.3.1. Predicting the Targets of AniHBr and Gastrointestinal Diseases

SwissTargetPrediction database (https://www.swisstargetprediction.ch/) was used to retrieve the targets associated with AniHBr, and collected all the targets with filter "Probability > 0".

We searched potential targets of Gastrointestinal Diseases by using "Gastrointestinal Diseases" as the key words in GeneCards database (https://www.genecards.org/) and set "Relevance score ≥10" as a filter.

2.3.2. Prediction of Candidate Targets and Construction of PPI Network

We used Venny 2.1.0 database (https://bioinfogp.cnb.csic.es/tools/venny/) to match the candidate targets of AniHBr and the potential targets of Gastrointestinal Diseases, and the intersection target genes were the potential targets in the inhibitory effect of AniHBr on Gastrointestinal Diseases.

The intersection targets were uploaded to String database (https://cn.string-db.org/) to build the PPI network interaction. PPI network was constructed and visualized by using Cytoscape 3.9.1. Then, we used CytoHubba to analyze topology parameters of each target and used the "Degree" value as a reference for the importance of the core targets.

After that, we put the corresponding 10 core targets into Cytoscape 3.9.1 to construct the relationship network between each 10 core targets.

2.3.3. GO Biological Process and KEGG Pathway Enrichment Analysis

We uploaded intersected target genes to DAVID database (https://david.ncifcrf.gov/) to perform GO biological process and KEGG pathway enrichment analysis ($P < 0.05$). Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) analysis were involved in GO enrichment analysis. We used the bioinformatics resource KEGG to mine significantly altered metabolic pathway enriched in the gene list.

2.3.4. Cell Culture

SD rat colonic smooth muscle cell line IM-R069 was bought from Xiamen Immocell Biotechnology Co. Ltd., China. and cultured in IM-R069-1 media (Immocell, Xiamen, China) under the condition of 37 °C and 5% CO_2 in the air.

2.3.5. AniHBr Preparation

AniHBr was dissolved in deionized water[17] by sonication to prepare a 5 mg/mL stock solution, which was then filtered through a 0.22 μm filter and stored at -80°C.

2.3.6. Reverse Transcription Quantitative Real-Time PCR

RNA was extracted from IM-R069 cells treated 24h following AniHBr (0, 10, 20 ug/mL) treatment using TRIzol from Invitrogen (Thermo Fisher Scientific, MA, United States) and subsequently reverse-transcribed using the PrimeScript™ RT reagent Kit (Takara). Real-time polymerase chain reaction was conducted according to established protocols from prior studies, and data were analyzed utilizing the $2^{-\Delta\Delta CT}$ method.[29] Primer sequences for the target genes are listed as follow.

Gene	Forward (5′-3′)	Reverse (5′-3′)
EGFR	TGCACCATCGACGTCTACAT	AACTTTGGGCGGCTATCAG
STAT3	CACCCATAGTGAGCCCTTGGA	TTTGAGTGCAGTGACCAGGACAG
GAPDH	GCTGAGAATGGGAAGCTGGT	CCTTGGCAGCACCAGTGGAT

2.3.7. Western Blot

After 24h treated with AniHBr (0, 10, 20 ug/mL), total proteins were extracted using RIPA buffer (Beyotime, Shanghai, China) according to the manufacturer's instructions. The protein lysate was subjected to electrophoresis and transferred onto a PVDF membrane (Bio-Rad, CA, United States). The following primary antibodies sourced from HUABIO (Zhejiang, China): p-AKT (1:5000; ET1607-73), AKT (1:5000; ET1609-47), GAPDH (1:5000; R1108-1). And p-PI3K (1:1000; AF3242) from Affinity (Suyang, China), PI3K (1:1000; A28092) from ABclonal (Wuhan, China) were used to incubate the membrane, respectively. The proteins were detected using ECL Plus Reagent (Beyotime) and quantified using Image Lab software.[29]

2.4. Statistical Analysis

All results are expressed as means ± SD (Standard Deviation) and data was analyzed by one-way analysis of variance (ANOVA) with SPSS software (version 25, IBM, USA). P values < 0.05 were considered significant.

3. Results

3.1. AniHBr Relaxed Ach-Induced Contraction of Isolated Rat Intestinal Smooth Muscles

Figure 1A is waveform about strain of the isolated intestinal segments. For ACh-induced spasm of isolated rat intestinal smooth muscles, AniHBr (0.5 μg/mL, 1.0μg/mL, 2.0 μg/mL) significantly reduced the average strain ($P<0.01$ vs. control) (Figure 1B-D) in a time-dependent manner within 2 min. While the relaxation effect of AniHBr on isolated intestinal smooth muscles was independent on concentration ($P>0.05$ vs. control) (Figure 1E and F) at the same time point.

3.2. AniHBr Relaxed Ach-Induced Contraction of Isolated Rabbit Intestinal Smooth Muscle

Similar with rat intestinal smooth muscles, AniHBr (0.25μg/mL, 0.5μg/mL, 1.0μg/mL) suppressed the ACh-induced contraction of isolated rabbit intestinal smooth muscles with significantly reducing the average strain ($P<0.01$ vs. control) (Figure 2B-D) in a time-dependent manner within 2 min. The relaxation effect of AniHBr on the contraction of isolated intestinal smooth muscles of rabbit was still independent on concentration ($P>0.05$ vs. control) (Figure 2E and F).

3.3. Pharmacokinetic Parameters in Beagle Dogs Following Oral Administration of AniHBr

After oral administration of AniHBr, absorption occurred rapidly and the plasma concentrations remained quantifiable up to 8 h post-dose (Figure 3). The peak plasma concentration (C_{max}, 51.01± 37.99 ng/mL) was observed at 0.72±0.63 h (T_{max}) post-dose (Table 1). The clearance (Cl/F) and volume of distribution (Vd/F) for AniHBr were 75843±30916 mL/h and 109324±39663 mL, respectively. Following a single oral dose of AniHBr, the area under the plasma concentration-time curve (AUC) including mean $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ values were determined. The mean $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ values were 74.21±30.90 h*ng/m, 75.58±31.17 h*ng/m, respectively. After oral administration, AniHBr distributed with a rate constant of the process (λz) of 0.70±0.13 h^{-1} and a half-life ($T_{1/2\ (d)}$) of 1.02±0.19 h.

3.4. Targets of AniHBr and Gastrointestinal Diseases

In SwissTargetPrediction database, we collected 165 potential targets for AniHBr, and in GeneCards database, through the key word "Gastrointestinal Diseases", we obtained 9047 potential targets. Moreover, we matched the candidate targets of AniHBr and Gastrointestinal Diseases obtained; 148 genes were obtained which might be potential targets in the inhibitory of AniHBr on contraction of intestinal smooth muscles (Figure 4A).

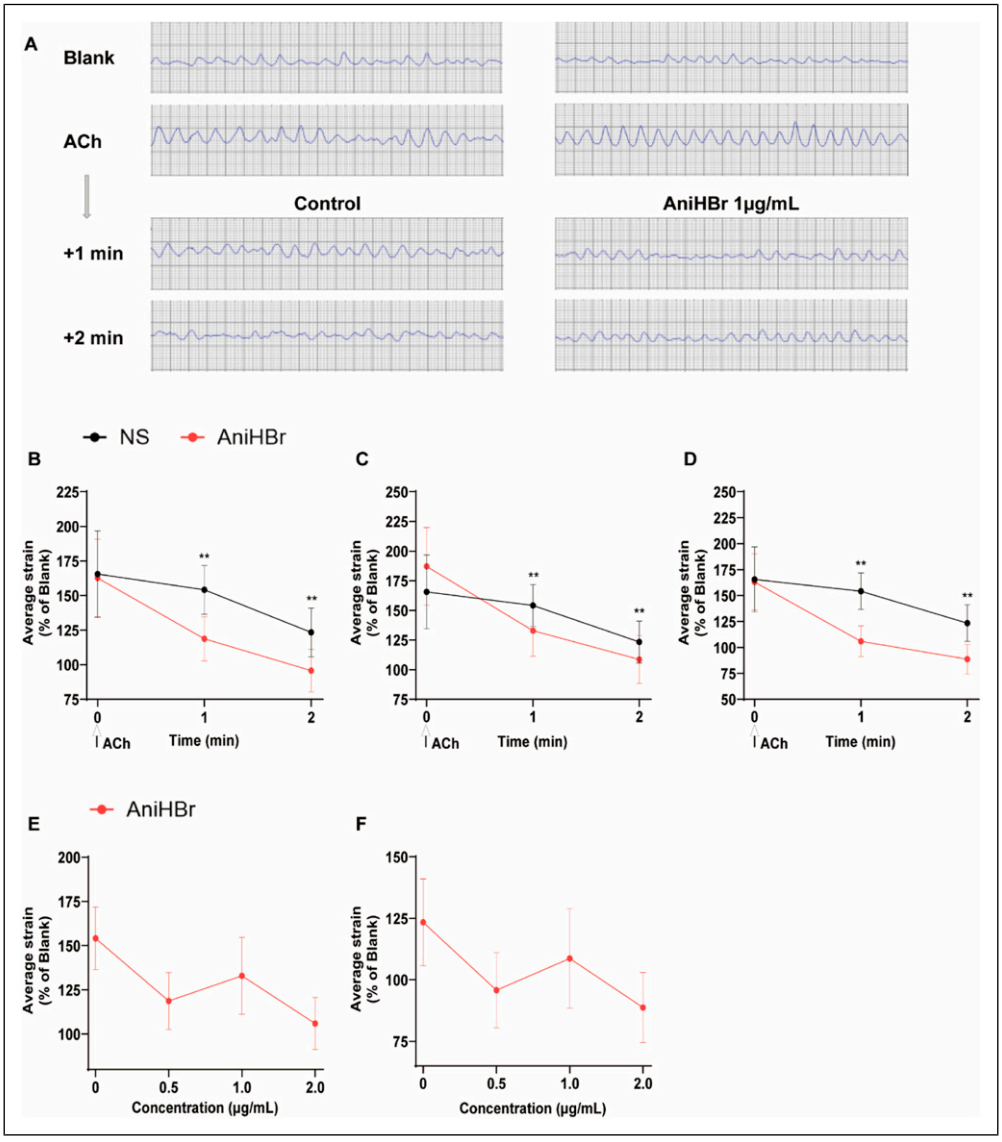

Figure 1. Inhibitory effect of AniHBr on Ach-induced contraction of isolated rat intestinal smooth muscles. (A) Waveform diagram for the inhibitory effect of AniHBr on Ach-induced contraction of rat intestinal smooth muscles, the intestinal smooth muscles were precontracted with ACh (1×10^{-6}moL/L). Then, normal saline, AniHBr were added respectively to analyze the parameters of contractive strain. (B)-(D) Average strain (g tension) of isolated intestinal smooth muscles in rats after the administration of 0.5 μg/mL (B), 1.0 μg/mL (C), and 2.0 μg/mL (D)AniHBr. (E and F) Concentration-response curves of AniHBr administration for 1 min (E) and 2min (F) Mean ± SD, n=10, $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Control

3.5. Construction of PPI Network

As shown in Figure 4A, we identified the PPI network of the 148 intersected target genes through String database. After uploading the PPI network shown in the above image to Cytoscape 3.9.1, we examined each target's topological parameters using the Network Analyzer feature of the program (Table 2). Based on the ranking of Degree value, the top 10 are selected as the core targets: EGFR, STAT3, SRC, MAPK1, PIK3CA, NOS3, PIK3R1, KDR, NFKB1, PARP1. Meanwhile, we used the Cytoscape 3.9.1 to construct the relationship network between the top 10 core targets. The visualization results are shown in Figure 4B and C.

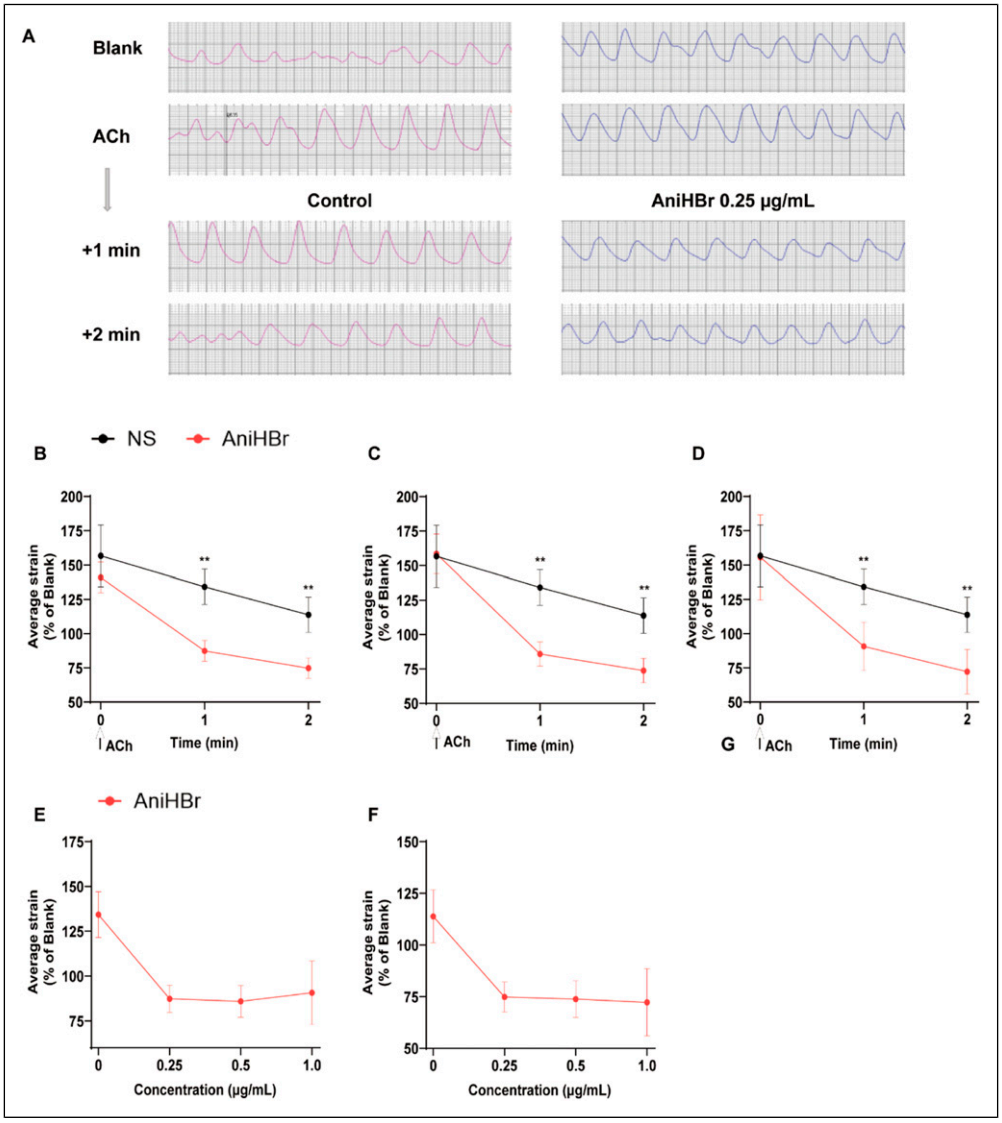

Figure 2. Inhibitory effect of AniHBr on Ach-induced contraction of isolated rabbit intestinal smooth muscles. (A) Waveform diagram for the inhibitory effect of AniHBr on Ach-induced contraction of rabbit intestinal smooth muscles, the intestinal smooth muscles were precontracted with ACh (1×10^{-6}moL/L). Then, normal saline, AniHBr were added respectively to analyze the parameters of contractive strain. (B)-(D) Average strain of isolated intestinal smooth muscles in rabbits after the administration of 0.25 μg/mL (B), 0.5 μg/mL (C), and 1.0 μg/mL (D)AniHBr. (E) and (F) Concentration-response curves of AniHBr administration for 1 min (E) and 2min (F) Mean ± SD, n=10, $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Control

3.6. GO Biological Process and KEGG Pathway Enrichment Analysis

To investigate the mechanisms, we uploaded 148 intersected targets into DAVID database for GO and KEGG pathway enrichment analyses. Figure 4E showed that A total of 1677 GO functional items were obtained, including 1398 biological processes (BP), 104 cellular components (CC) and 175 molecular function (MF), which mainly involved positive regulation of MAPK cascade (BP), response to peptide (BP), response to oxidative stress (BP), synaptic membrane (CC), neuronal cell body (CC), membrane raft (CC), protein serine/threonine/tyrosine kinase activity (MF), protein serine/threonine kinase activity (MF), protein serine kinase activity (MF), etc.

The results of KEGG pathway enrichment analysis showed that there be 133 major signaling pathways. As shown in Figure 4E, the high-ranking enriched pathway involved neuroactive ligand-receptor interaction, cAMP signaling pathway, Calcium signaling pathway, Ras signaling pathway, and PI3K-AKT signaling pathway so on.

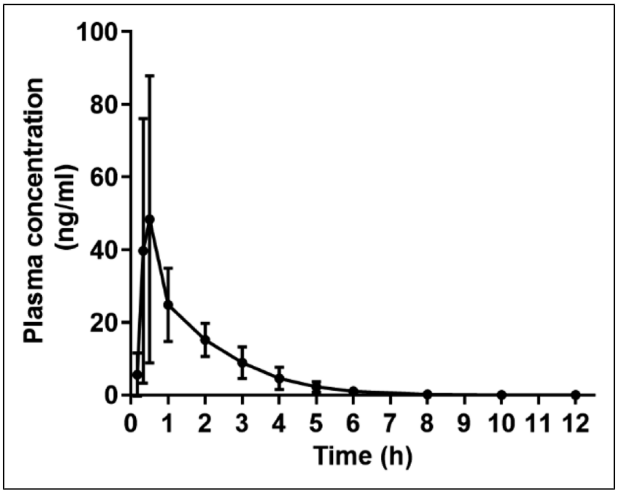

Figure 3. Plasma drug concentrations in Beagle dogs following single oral administration of 5 mg AniHBr. Blood sampling was performed at 0.17h,0.33 h,0.5 h,1 h,2 h,3h,4 h,5 h,6 h,8 h,10 h,12 h and 24 h, respectively. Mean ± SD, n=6

Combined with the finding of PPI network, most of the 10 core genes were related to Ras signaling pathway and PI3K-AKT signaling pathway, which might be the important mechanism of Ach-induced contractions of intestinal smooth muscles.

3.7. AniHBr Could Regulate the mRNA Expression of EGFR and STAT3 and the PI3K-Akt Signaling Pathway

To further validate the findings from network pharmacology, Reverse transcription quantitative real-time PCR and western blotting experiments were performed. As shown in Figure 5A and B, treatment with AniHBr for 24 hours increased the expression of EGFR and STAT3 at the mRNA level, and a concentration of 20 μg/mL AniHBr significantly upregulated the mRNA expression of both EGFR and STAT3 ($P<0.01$). Furthermore, we examined the protein levels of two key molecules in the PI3K-AKT pathway. As shown by the Western blot results, 10 μg/mL AniHBr significantly reduced the phosphorylation level of AKT, while 20 μg/mL AniHBr significantly decreased the phosphorylation levels of both PI3K and AKT. Thus, AniHBr may exert its regulatory effects by modulating the expression of EGFR and STAT3, as well as by inhibiting the PI3K-AKT signaling pathway.

4. Discussion

Intestinal smooth muscle spasm is commonly due to the contraction of intestinal smooth muscle caused by the excitation of parasympathetic nerve and subsequent release of excitatory neurotransmitter Ach.[30] ACh is an excitatory neurotransmitter of the parasympathetic nervous system, which could induce the contraction of intestinal smooth muscle through the activation of muscarinic receptors.[31,32] RaceAni could inhibit ACh-induced endothelium-dependent relaxation by inhibiting mAChRs,[33] which is possibly associated with the inhibition of nitric oxide production.[18] Cigarette smoke increased the

Table 1. AniHBr Pharmacokinetic Parameters in Beagle Dogs After Single Oral Administrations of 5 mg AniHBr (n=6)

Parameter	AniHBr
C_{max} (ng/mL)	51.01 ± 37.99
$AUC_{0\text{-}t}$ (h*ng/mL)	74.21 ± 30.90
$AUC_{0\text{-}\infty}$ (h*ng/mL)	75.58 ± 31.17
$T_{1/2\ (d)}$(h)	1.02 ± 0.19
λ_d (1/h)	0.70 ± 0.13
T_{max} (h)	0.72 ± 0.63
Vd/F (mL)	109324 ± 39663
Cl/F (mL/h)	75843 ± 30916

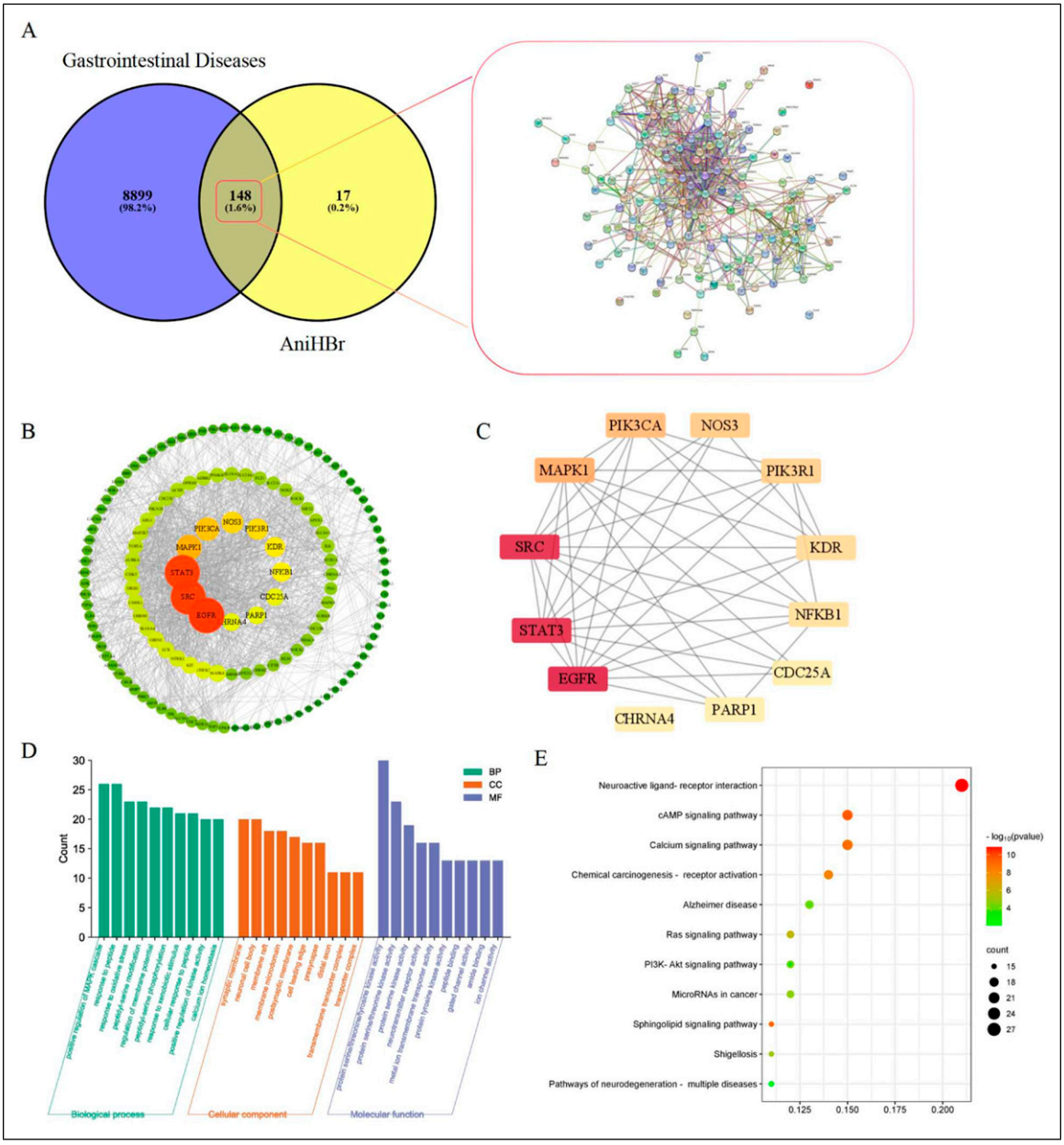

Figure 4. Target screening, establishment of PPI network and GO biological process and KEGG pathway enrichment analysis. (A) Potential targets of AniHBr inhibition on gastrointestinal diseases and PPI network. (B) PPI network of the intersected genes. (The color of the node is marked from red to green based on the Degree value in descending order.) (C) Relationship network between core targets of AniHBr. (The color of the node is marked from red to yellow based on the Degree value in descending order.) (D) The bubble diagram of GO enrichment analysis of 148 intersection targets, including the top 10 significant enrichment terms of BP, CC and MF. (F) The bar plot diagram of KEGG pathway enrichment analysis of 148 intersection targets (top 11)

expression of mAChR M2 and M3 in airway smooth muscle cells, while RaceAni inhibited cigarette smoke-induced airway smooth muscle cell proliferation and tracheal smooth muscle constriction.[34] Consistent with the relaxation of smooth muscles by RaceAni, the present study reported that AniHBr inhibited ACh-induced contraction of isolated intestinal smooth muscles by reducing the average strain in a time-dependent manner within 2 min.

In the present study, the doses of AniHBr in isolated rat intestinal smooth muscles (0.5~2 μg/mL) were different with that of Rabbits (0.25-1 μg/mL), those doses are determined by pre-experiments (0.25-4 μg/mL for both animals). RaceAni is a chiral drug that has two pairs of enantiomers.[35] The relative amount of the isomers (6R, 2′S), (6S, 2′R), (6S, 2′S), and (6R, 2′R) in RaceAni was 30%, 30%, 20%, and 20%, respectively. Among them, the 6R, 2′S configuration is responsible for the

Table 2. Topological Parameters of Candidate Targets

Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree
EGFR	50	TOP2A	15	PLG	11	TRPA1	7	TRIM24	5	CA2	2
STAT3	49	AURKA	15	CHRNB4	10	DRD5	7	PDE11A	4	KCNJ1	2
SRC	49	MAP2K7	15	CHRM2	10	PDGFRA	7	MPI	4	SCD	2
MAPK1	32	ACHE	14	CFTR	10	ABCC1	6	ADRB3	4	CNR2	2
PIK3CA	29	ADRB2	14	NFE2L2	10	SPHK1	6	PIM1	4	MAN2B1	2
NOS3	26	CDC25C	14	BLM	10	HRH3	6	SLC5A1	4	PTGS1	2
PIK3R1	25	OPRM1	14	HDAC6	10	NTSR2	6	FPR2	4	SLC6A5	2
KDR	23	PIK3CD	14	ROCK2	10	ADORA1	6	KCNA5	4	PDE9A	2
NFKB1	22	RPS6KB1	14	HTR2C	9	OPRD1	6	SCN3A	4	SLC5A4	2
PARP1	19	SLC18A3	13	FPR1	9	CACNA1B	6	HTR4	3	NUDT1	1
CHRNA4	19	SLC6A2	13	CDC7	9	CTSD	6	TKT	3	DCUN1D1	1
CDC25A	19	SLC6A3	12	ITK	9	HTR1D	6	MTAP	3	DPP8	1
KIT	18	APEX1	12	GSTP1	8	LGALS3	6	CCR1	3	DPP9	1
MAPK8	18	ROCK1	12	NTRK3	8	HDAC5	6	PSMB9	3	HRH4	1
CHEK2	18	ILK	12	MMP7	8	KDM1A	6	GPR55	3	MAN2A1	1
LCK	17	SIRT2	12	CHUK	8	NR1I2	5	PRCP	3	LNPEP	1
NTRK1	17	SLC2A1	12	PRKCI	8	PNP	5	GLB1	3	MMP12	1
GRIN1	17	FLT1	12	TLR9	8	MGEA5	5	SLC5A2	3	PABPC1	1
SLC6A4	16	NOS2	12	CYP3A4	7	CSNK1D	5	KLF5	3	TFPI	1
DRD2	16	CHRNA3	11	PTPN2	7	EHMT1	5	MMP13	3	PNMT	1
CHRM1	16	CDC25B	11	ADAM10	7	CAPN1	5	OPRK1	3		
CHEK1	16	AURKB	11	ADK	7	HDAC7	5	ACVRL1	2		
ABL1	15	HTR3A	11	PDE5A	7	CHRM3	5	AOC3	2		
CDK5	15	MAPK9	11	TLR8	7	SIGMAR1	5	C5AR1	2		

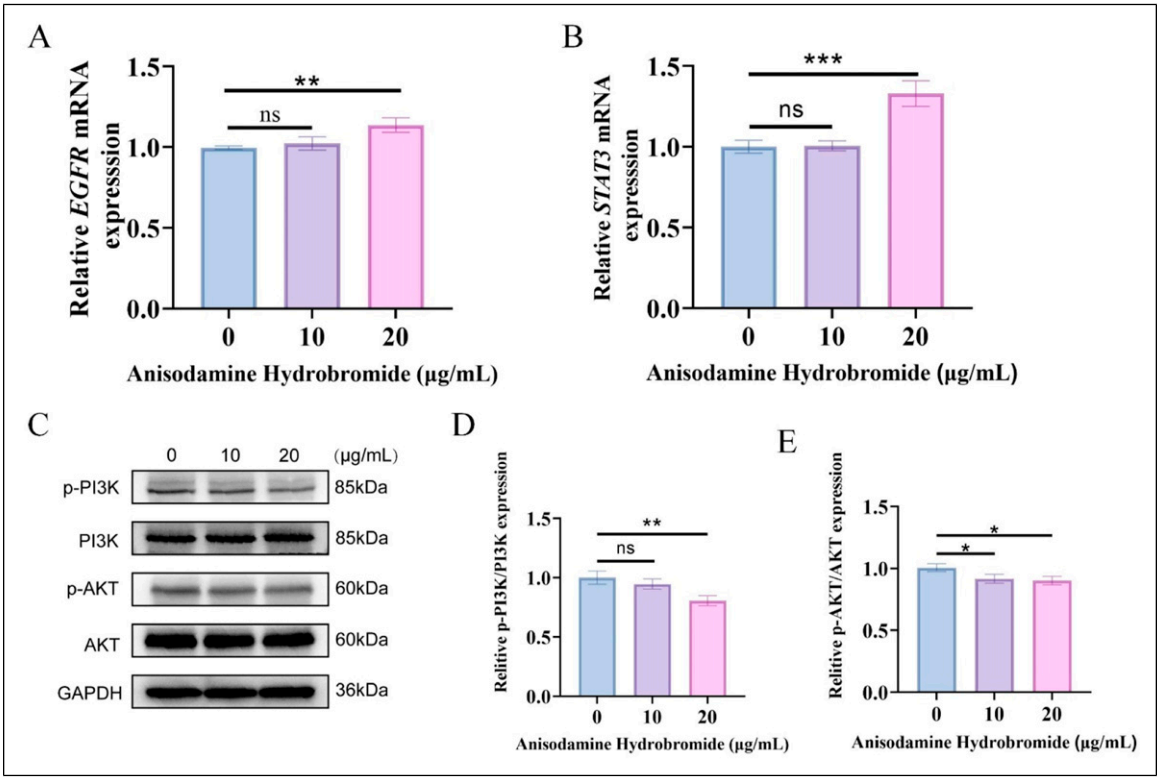

Figure 5. AniHBr increased the mRNA levels of EGFR and STAT3, and suppressed PI3K-Akt signaling pathway. The mRNA expression of EGFR (A) and STAT3 (B) in IM-R069 cells from 0 ug/mL, 10ug/mL, and 20 ug/mL AniHBr 24h treatment. (C) Representative immunoblots for p-PI3K, PI3K, p-AKT, AKT and quantitative analysis of (D) p-PI3K and (E) p-AKT in IM-R069 cells. Mean ± SD, n=3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. 0 ug/mL

antispasmodic effect of RaceAni,[15] accounting for about 30% in RaceAni. Different isomers often exhibit different pharmacological activities and even opposite physiological effects.[36] It was previously reported that RaceAni was more toxic than AniHBr.[14] The stronger toxicity of RaceAni may be derived from the mixture of isomers, while high purify of AniHBr could diminish the toxicity. It is thought that AniHBr exerts its effect on the abdominal ganglion mainly by inhibiting mAChRs.[37] However, the specific mAChR involved in the antispasmodic effect of AniHBr should be studied in the future. In the pharmacokinetic experiments, one notable observation was the considerable inter-individual variability in the plasma concentrations of AniHBr. This variability could potentially be attributed to factors such as differences in individual absorption. We acknowledge that the current study represents a preliminary pharmacokinetic investigation. Further studies are warranted to elucidate the underlying mechanisms contributing to this variability.

"Network pharmacology" was first proposed in 2007, the same year Chinese scholars studied traditional Chinese medicine prescriptions *via* biological networks. Recent advances in systems biology have driven its application in traditional Chinese medicine, yielding series of achievements.[38] In addition, network pharmacology combined with real-world adverse event reporting systems has been successfully applied to investigate drug-induced adverse reactions, such as drug-induced pancreatitis.[39] This approach provides a valuable reference for the safety evaluation of drugs like AniHBr. Enteric nervous system (ENS) was related to various of Gastrointestinal Diseases, in which ACh is a critical Neurotransmitters. Therefore, we chose Gastrointestinal Diseases as the keyword for the study of network pharmacology to predict the potential mechanisms of the inhibitory effect of AniHBr against Ach-induced contractions of intestinal smooth muscles.[40] According to the results of network pharmacology, both Ras signaling pathway and PI3K-AKT signaling pathway involvement in gastrointestinal physiology and pathophysiology,[41,42] leading to the way to further mechanism investigation.

Muscarinic receptors consist of five main subtypes, among which the gastrointestinal tract is primarily associated with the M1 and M3 subtypes. Ach-induced signal transduction is mainly mediated by the M3 subtype. Moreover, the M3 subtype receptor exerts its effects by regulating downstream signaling molecules or kinases including PI3K-AKT signaling pathway.[43] The PI3K-AKT signaling pathway is associated with a wide range of diseases, including cancer where sodium homeostasis and related transporters have been shown to play a regulatory role.[44] Inhibition of this pathway can often suppress tumor growth[45] and contribute to ameliorating conditions such as colitis.[46] In our research, we found that 20 ug/mL

AniHBr could both inhibit the phosphorylation levels of both PI3K and AKT, indicating the inhibitory effect of AniHBr against contraction of intestinal smooth muscles induced by ACh might be through PI3K-AKT signaling pathway.

There are several limitations in this study. Firstly, the inhibitory effect of AniHBr was studied in isolated rat and rabbit intestinal smooth muscles, but the pharmacokinetics were studied in beagle dogs. In addition, a single oral dose of 5mg AniHBr was administered to beagles, it is still unclear the precious concentration of AniHBr for human smooth muscles. Nevertheless, the specific oral dose for human needs further preclinical research.

5. Conclusion

In conclusion, AniHBr exhibits an obvious inhibitory effect on Ach-induced contraction of isolated rat and rabbit intestinal smooth muscles. The detailed pharmacokinetics of AniHBr in beagle dogs provided valuable information for clinical application of AniHBr. Furthermore, the regulatory effects of AniHBr might mediate the EGFR, STAT3 and PI3K-AKT signaling pathway.

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Ethical Considerations

All experimental procedures involving animals were approved by the animal committee of Sichuan Industrial Institute of Antibiotics (No. SYXK 2019-021).

Author Contributions

This manuscript was conceptualized by all the authors. Study conception and design: Feng Wan, Huali Fan and Cheng Peng; investigation: Feng Wan, Heng Zhang; data collection: Feng Wan, Ye Zeng and Wenli Jiang; analysis data: Ye Zeng an Qiang Cheng; draft manuscript preparation: Feng Wan and Huali Fan; editing & reviewing: Cheng Peng.

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Declaration of Conflicting Interests

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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