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# Andrographolide Alleviates S. *Pneumoni*ae Bacterial Meningitis as an NF-κB Pathway Inhibitor and Antibiotic

Yuan Chang

School of Life Science, Nanjing University, Nanjing, Jiangsu 210046, China.

## Abstract

**Meningitis is a severe inflammatory response in the central nervous system (CNS). Patients may suffer from irreversible damages to their brains. This research finds a new way to cure meningitis since the overactivation of the NFkB pathway results in severe meningitis inflammatory response. And andrographolide (andro) can inhibit the canonical NFkB pathway by blocking the phosphorylation and translocation of p65. Together with its antibiotic ability, andro is theoretically suitable for curing bacterial meningitis. No one has tried to use andrographolide to alleviate meningitis, this work designed experiments to test the ability of andrographolide.**

## Keywords

**Andrographolide, Bacterial meningitis, NF-kB pathway, S. Pneumoniae.**

## 1. Introduction

Meningitis is a severe inflammatory response in the meninges region caused by various reasons. There are two kinds of meningitis, aseptic meningitis, and bacterial meningitis. Although aseptic meningitis accounts for 85-95% of meningitis cases, it only has a mortality rate of 0.5-3% with few sequelae [1]. For bacterial meningitis, on the other hand, the mortality rates are approximately 10-15%, even higher in developing regions [2]. A considerable part of those who recover from meningitis suffered from irreversible damages to their brain, causing seizures and other function losses [3]. The standard treatment for bacterial meningitis including vaccines and antibiotics [4,5], which both aim to cure bacterial meningitis because of huge individual differences existing in aseptic meningitis.

The predominant microbes causing bacterial meningitis are S. agalactiae, N. meningitidis, E. coli (common in neonates), and L. monocytogenes (common in neonates and the elderly) [5]. The Neisseria meningitidis is more infective especially in Africa and has specific vaccines according to WHO's websites about meningitis. But the mortality rates of N. meningitidis 3-13% are much less than meningitis triggered by S. pneumoniae(Sp) which has mortality rates of 19-26% [1]. So, this article focuses on S. pneumoniae meningitis.

There are several pathways possible for bacterial to invade into the cerebral region [6]: (1) after sepsis, bacteria flow with blood, trespass blood-brain-barrier (BBB) and infect the meninges; (2) the bacteria are transported intracellularly or paracellularly through olfactory nerve cells in nasal ethmoid or other peripheral nerves cells like trigeminal nerve cells. However, BBB is a tight junction between endothelium cells, sealing up overlapping areas of astrocyte foot process and endothelial cells, that only allow some restricted substances to enter the cerebral region and prevent harmful substances and bacteria from entering in most circumstances [7]. And the nerve pathway is also hard for bacteria to pass through. So in most cases, the brain is safely taken care of. But once the protectors fail to keep the bacteria out, the infection will occur, followed by inflammation, BBB permeability increase, and leukocyte transmigration [2].

It has been proved that the NF-kB pathway plays a key role in inflammation processes. Over-activation of the NF-kB pathway results in cell dysfunctions and other disadvantages in disease

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treatment [8]. The activation of NF-kB pathway has the following steps [8,9] (Fig.1): (1) proinflammatory cytokines (TNFs, IL-1, etc.) and TLRs binds to receptors on the cell membrane and triggers the canonical NFkB pathway; (2) IkB kinase (IKK) is activated; (3) IKK phosphorylates IkB; (4) IkB disassociates with heterodimer complex of p65/p50; (5) the heterodimer translocates into the nucleus and binds to DNA; (6)the heterodimer acts as a transcription factor and regulates expression of inflammatory cytokines (IL-8, TNF-α, etc.).The alternative pathway consists of different IKK subunits and heterodimer complex subunits [8]. The alternative pathway activation is held to a low level due to the controlled expression of NIK [10]. But we should not neglect the compensation effect of the alternative pathway when the canonical pathway is inhibited. The p100 protein, also known as nuclear factor kappa B subunit 2, is specific to the alternative NFkB pathway. The p100 is coded by an 8420nt gene with 25 exons located at 10q24.32 in humans [11] or an 8644nt gene with 23 exons located at 1q54 in rat [12]. By knocking out the p100 gene, the alternative NFkB pathway is blocked, leaving only the canonical NFkB pathway operating in the rat.

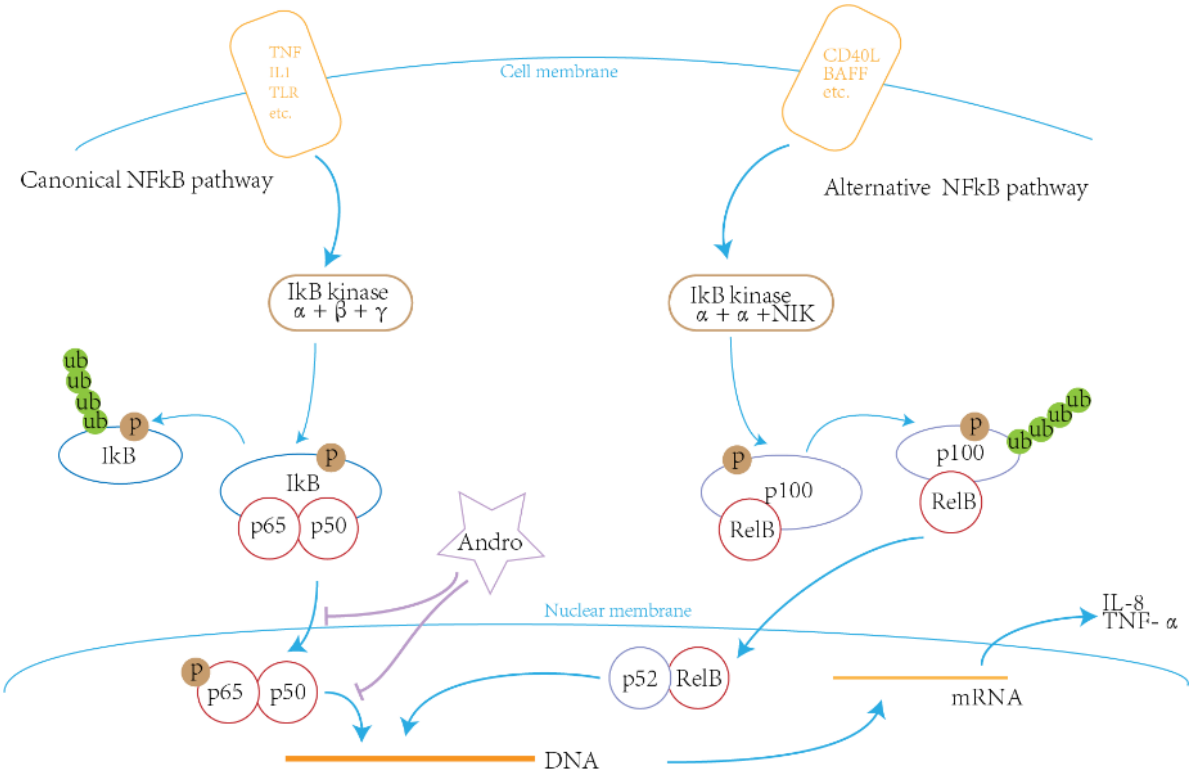


Fig 1 NF-kB pathway activation and inhibition.

Several genes are downstream genes of the NFkB pathway. Transcriptions and translations of these genes have different functions in cells. IL-8 is crucial for cell survival. IL-1β and TNF-α are positive feedback proteins of the NFkB pathway. They are also inflammatory cytokines. IkBα and A20 are negative feedback signal proteins of the NFkB pathway.

White blood cell count (WBC) is a crucial indicator of meningitis. Meningitis is accompanied by increased permeability of the BBB, allowing white blood cells to enter the cerebrospinal fluid (CSF) [6]. Inflammation cytokines attract white blood cells to penetrate the BBB into the CSF. In the initial stage of meningitis, WBC is higher than normal. Extra white blood cells help to kill and remove the S.p that infected the meninges. In the later stages of meningitis, bacteria amount, and inflammation cytokine level are reduced. Consequently, the WBC decreases, and white blood cells leave the CSF. A decrease in WBC means alleviation of meningitis.

It's proved that inhibiting the NF-kB pathway can alleviate bacterial meningitis inflammation, lower the mortality rate, and avoid further damage to the brain. For example, the p65 subunit has a

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transcriptional transactivation region, which the p50 subunit doesn't have, that can bind to DNA and regulate inflammatory cytokines expression [13]. So, by knocking out the p50 gene, the p50(-/-) mouse has p65/p65 instead of p65/p50 complex. Lacking p50 results in overactivation of the NF-kB pathway, which contributes to a higher mortality rate [14]. On the contrary, with the calpain inhibitor, I N-acetyl-leucinyl-leucinyl-norleucinal(ALLN) interfering with the IkB proteolysis, or BAY 11-7085(BAY) inhibiting IkB phosphorylation, IkB can no longer disassociate with p65/p50 heterodimer, causing downregulation of NF-kB pathway, leading to alleviation of inflammation and other meningitis conditions [15].

Andrographolide (Andro) is a diterpenoid lactone extracted from the plant *Andrographis paniculata* [16]. Andro shows potential both in blocking the NF-kB pathway and inhibiting bacterial growth. Andro can downregulate the NF-kB pathway by interfering subunits p50, p65 [16], and p38 [17,18]. Andro formed a covalent adduct with p50 at the Cys62 residue making it unable to bind to DNA [19]. Andro also inhibited the phosphorylation of p65 Ser536 residue [18,20], which is required for translocation of p65 to the nucleus.

Andro is a good antibiotic. The minimum inhibitory concentration to *S. pneumoniae* of andro is 0.25~1mg/mL [21]. And andro is usually taken orally [16]. It has minimal toxicity: mouse can tolerate 5g/kg oral administration for 14 days and 500mg/kg for over 21 days [22]. The LD50 is 11.46g/kg for mice if intraperitoneally injected [23]. The over 10 times difference between minimum inhibitory concentration and bearable concentration gives us the possibility to use andro's antibiotic ability even though it will have to go pass barriers to enter the cerebral region.

Yet, scientists do not know how andro will perform in the cerebral area inhibiting the NF-kB pathway, disinfecting *S. pneumoniae*, and alleviating meningitis inflammation. Nearly no research has been carried out using andro for meningitis. So, the proposal conducts this experiment based on andro's ability to inhibit infection and to regulate the NFkB pathway.

This proposal predicts that andrographolide administered intraperitoneally can dose-dependently reduce NFkB activation and eliminate *S. pneumoniae* which causes meningitis in CSF. Firstly, it's needed to test that whether, in CSF, andro inhibits the canonical NFkB pathway in our S.p triggered meningitis rat models. And the alternative NFkB pathway is neglectable. Since the NFkB p100 subunit is needed to form a transcription factor in alternative NFkB pathway, this proposal uses knock-out rats p100(-/-) to ensure no alternative NFkB pathway would stand in the way. Secondly, this proposal plans to use western blot to analyze the levels of IKK, pIkB, pp65(Ser536), p65, p50 in the cytoplasm, and pp65(Ser536), p65, pp50 in the nucleus. The proteins analyzed by western blot is denatured while testing. This proposal also plans to use the NFkB p65 transcription factor reporter assay kit (an ELISA kit) to analyze the NFkB DNA-binding activity in nucleus protein. When the p65 binds to DNA, the p65 will transform into another allosteric conformation which has a specific domain. The specific domain binds to the antibody in the ELISA kit. So, the nucleus protein tested by this kit is not denatured. It can be tested whether andro successfully blocks NFkB p65 phosphorylation and translocation through this experiment. Thirdly, using rt-qPCR, this proposal can analyze the levels of mRNA transcription of IL-8, IL-1β， TNF-α, and IkBα. Then, white blood cells (WBC) are counted. Last but not least, GFP fluorescent *Streptococcus pneumoniae* is used for bacteria count. The positive antibiotic control drug will be vancomycin which is a commonly used drug for treating meningitis [4]. The positive inhibitor control drug will be ALLN and BAY. The negative control group will be treated with solution DMSO.

## 2. Materials and Methods

### 2.1 GFP Fluorescent S. pneumoniae

Kjos, M., et al developed a protocol to construct an *S. pneumoniae* strain with high fluorescent performance [24]. Briefly, the superfolder GFP gene (sfgfp) and chloramphenicol resistance gene (cat) were inserted into plasmids. After amplification, plasmids were transformed into *S. pneumoniae*,

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giving us *S. pneumoniae* with fluorescein expression. The chloramphenicol resistance gene is used for it will not reduce the effect of vancomycin.

Using a fluorescence detector, the relative concentration of *S. pneumoniae* is measured.

### 2.2 Animals

Male Wistar rats (350–400 g) adult wild-type rats and p100(-/-) rats were used.

### 2.3 Rat Model of S. pneumoniae Meningitis

The proposal sets 7 groups: (1) blank control group, wild type rats (wt), no *S. pneumoniae* infected (Sp-); (2) untreated group 1 (negative control), wt, *S. pneumoniae* infected(Sp+), no drug; (3) untreated group 2, p100(-/-), Sp+, no drug; (4) treated group 1, wt, Sp+, andro low concentration (andro+) and andro high concentration(andro++); (5) treated group 2, p100(-/-), Sp+, andro+ and andro++; (6) antibiotic positive control group, wt, Sp+, vancomycin+; (7) inhibitor positive control group, wt. Sp+, ALLN+, and BAY+. Vancomycin is used to treat bacterial meningitis in clinical. ALLN and BAY are two inhibitors of NFkB pathway. Vancomycin, ALLN, and BAY are introduced in the introduction.

Pneumococcal meningitis rat model is widely used. This proposal uses the rat model used by [15]. Briefly, adult male Wistar rats (both wild type experimental group and p100(-/-) experimental group) weighing 350–400 g were transcutaneously injected 150μL of $10^7$ cfu/mL of *S. pneumoniae* into the cisterna magna to induce meningitis. The control group (wild type) were injected 150μL DMSO. Each rat must be put into an individual cage. The clinical evaluation and scoring were done 24h after the injection. The following criteria were evaluated: "bodyweight loss; water and food uptake; tremor and piloerection; vigilance; motor skill; body temperature." The maximum total score was 16. The wild-type control group gets a score of 0.

All drugs (BAY 11-7085, ALLN, andro, vancomycin) are dissolved in DMSO with the same concentration.

A catheter was inserted into the cisterna magna for extracting CSF. Before ending the experiment, rats were deeply anesthetized with thiopental. Transcardially injection with 150 mL of ice-cold DMSO killed the rat. Brains were extracted and frozen in a tissue-freezing medium.

### 2.4 White Blood Cells Count

10μL of CSF from each group was extracted using the catheter. The CSF was diluted with DMSO. Hemocytometer is used to count the WBC.

### 2.5 S. pneumoniae Concentration Measurement

10μL of CSF from each group extracted using the catheter. The CSF was diluted with DMSO. The fluorescent brightness was measured using a fluorescence detector. By comparing the fluorescent brightness, the relative concentration of S.p is determined.

### 2.6 Western blot

The cytosolic and nuclear proteins are collected use methods mentioned in [25]. Briefly, cells were lysed in a cold hypotonic buffer for 15 min and vortexed for 10 s. Nuclei were extracted by centrifugation at 15,000 g for 1 min. A pellet containing nuclei was resuspended in a cold hypertonic buffer for another 30 min. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 g for 2 min.

Immunoblots methods were mentioned in [26]. IKK, pIkB, pp65, p65, p50 were separated by 10% SDS-PAGE gel. Anti-IKK, anti-pIkB, anti-pp65(Ser536), anti-p65, anti-p50 antibodies were used to analyze the levels of these proteins.

### 2.7 ELISA Assay

An NFkB p65 transcription factor reporter assay kit is used to analyze the NFkB DNA-binding activity following the instruction of the kit.

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### 2.8 rt-qPCR

IL-8, IL-1β, TNF-α, and IkBα are downstream factors of NFkB pathway. To directly analyze the expression level of NFkB pathway, it is needed to measure the mRNA level of IL-8, IL-1β, TNF-α, and IkBα.

rt-qPCR is used to analyze the transcription levels of IL-8, IL-1β, TNF-α, and IkBα. Briefly, mRNA was extracted and reverse transcripted to cDNA. cDNA was amplified and Quantitated in Realtime-PCR. β-actin was used as the internal control. The primers sequences are listed in Table.1.

### 2.9 HPLC-MS/MS

5μL CSF was extracted, filtrated, and measured by HPLC-MS/MS to determine the concentration of andro. The sample used in HPLC-MS/MS is andro dissolved in DMSO.

### 2.10 Statistical Analysis

(imaginary)Data are presented as means ± SEM. One-way ANOVA was used to determine significant differences between treatment groups. Significant levels were set at $P < 0.05$(*), $P<0.01$(**).

Table 1 Primers sequences of rt-qPCR

| ***Targets*** | **Primers sequences** | |
|---|---|---|
| | Forward | Reverse |
| *IL-8 (human) [27]* | 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' | 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' |
| *IL-1β (human) [28]* | 5'-TTTGAAGAAGAGCCCATCATCC-3' | 5'-CCAGCCAGCACTAGAGATTTG-3' |
| *TNFα (human) [29]* | 5'-CTCTTCTGCCTGCTGCACTT-3' | 5'-CAGCTTGAGGGTTTGCTACA-3' |
| *IkB (mouse) [30]* | 5'-CTACACCTTGCCTGTGAGCA-3' | 5'-TCCTGAGCATTGACATCAGC-3' |
| *β-Actin (both [27]* | 5'-TCATGAAGTGTGACGTTGACATCCGT-3' | 5'-CCTAGAAGCATTTGCGGTGCACGATG-3' |

## 3. Possible Results

### 3.1 Pre-test results:

#### 3.1.1 Andro is able to penetrate into CSF

Table 2 Pre-test result 1 The relationship between the injected Andro and the concentration of Andro in the CSF (Rat type: Wt, sp+, untreated.)

| | **Pre-test group 1** | **Pre-test group 2** | **Pre-test group 3** |
|---|---|---|---|
| *Andro injected/ mg/kg* | 0 (injected with vehicle DMSO) | + | ++ |
| *Andro concentration in CSF* | 0 | +* | ++* |

Note.1: * means significant compared to the group that was injected with DMSO. Note.2: + means low concentration. ++ means high concentration.

This pre-test result shows the relation between the amount of andro been injected intraperitoneally and the concentration of andro in CSF. With more andro been injected into rats, a higher concentration can be observed in CSF.

#### 3.1.2 Andro is not able to penetrate into CSF

Table 3 Pre-test result 2 The relationship between the injected Andro and the concentration of Andro in the CSF (Rat type: Wt, sp+, untreated.)

| | **Pre-test group 1** | **Pre-test group 2** | **Pre-test group 3** |
|---|---|---|---|
| *Andro injected/ mg/kg* | 0 (injected with vehicle DMSO) | + | +++ |
| *Andro concentration in CSF* | 0 | 0 | + |

This pre-test result also shows the relation between the amount of andro been injected intraperitoneally and the concentration of andro in CSF. Despite the different amounts of andro injected into rats, the concentrations in CSF show no significant difference.

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### 3.1.3 Alternative NFkB pathway can be neglected

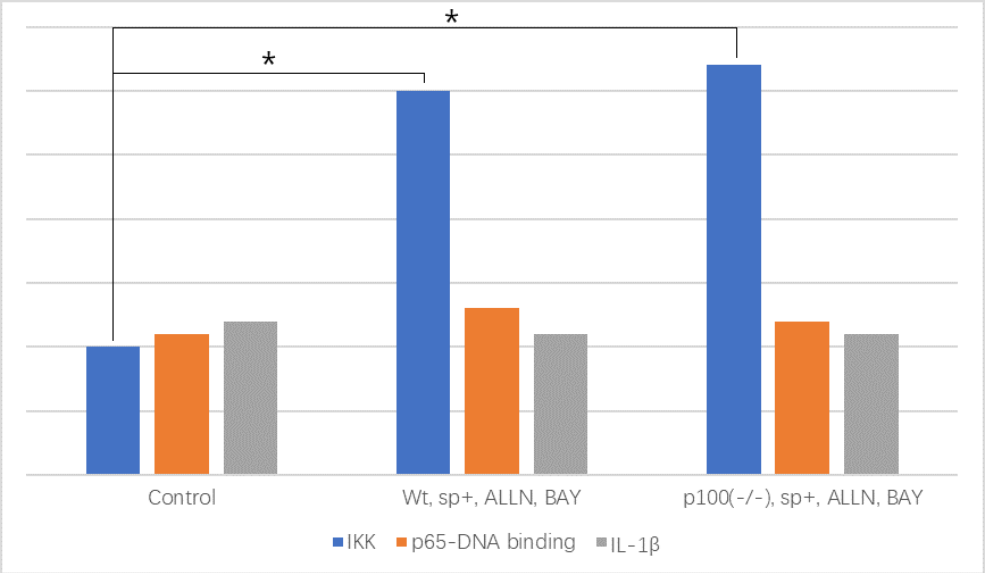


Fig 2 The ELISA result of IKK, IL-1β expressions, and p65-DNA binding activity.

IKK level is significantly higher in experimental groups. But p65-DNA binding activity and IL-1β expression show no significant difference.

### 3.1.4 Alternative NFkB pathway can not be neglected

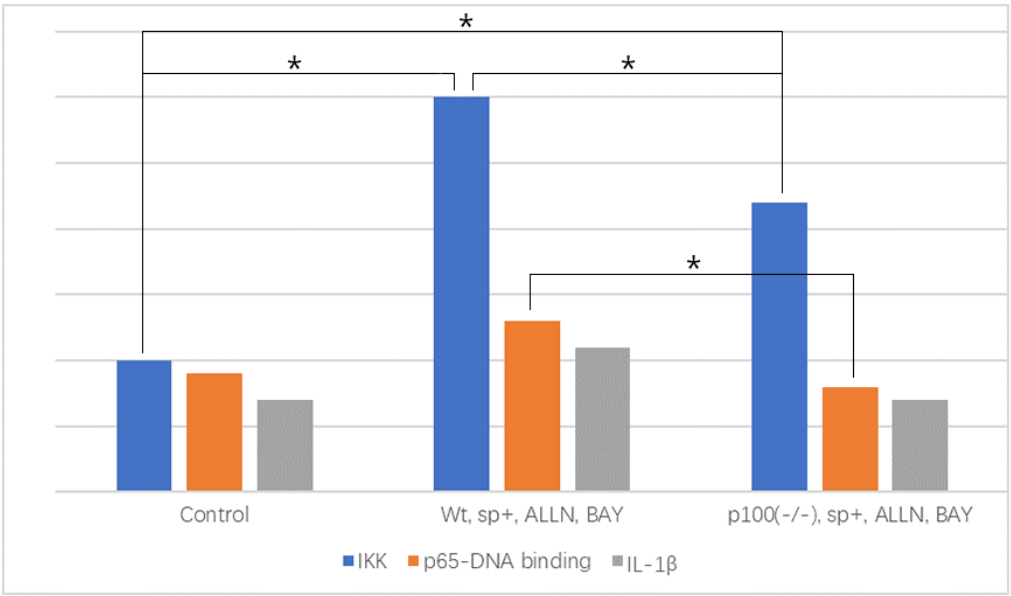


Fig 3 The ELISA result of IKK, IL-1β expressions, and p65-DNA binding activity.

p65-DNA binding activity and IL-1β expression between control and p100(-/-) group have no difference. IKK level is significantly higher in experimental groups. But IKK level and IL-1β expression in wt group are significantly higher than p100(-/-) group.

## 3.2 Results:

### 3.2.1 Andro dose-dependently inhibited NFkB p65 phosphorylation and translocation into the nucleus. p65-DNA binding activity was reduced. Meningitis inflammation was alleviated. Sp was killed.

White blood cells were counted after treatment is done. So, as described in the introduction, white blood cells finished its work killing bacteria and moved out of the CSF. So, lower WBC means after the treatment, the inflammation and infection were alleviated. Clinical scores are always higher than

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the blank control group because meningitis always causes damages to the rats. (More + means higher concentrations/level/scores. Green means the concentration/level/scores are low. Yellow means medium. And red means high.)

Table 4 Possible result 1 of each factor of rats in each group

| | Wt, sp-, DMSO | Wt, sp+, DMSO | Wt, sp+, andro+ | Wt, sp+, andro++ | Wt, sp+, ALLN, BAY | Wt, sp+, Vancomycin | |
|---|---|---|---|---|---|---|---|
| *IKK, IkB* | - | ++ | ++ | ++ | +++ | + | |
| *p65, pp65, p50 levels (cytoplasm)* | - | ++ | ++ /-(pp65) | ++ /-(pp65) | - | + | ALLN interferes with the IkB proteolysis. BAY inhibits IkB phosphorylation. |
| *p65, pp65, p50 levels (nucleus)* | - | ++ | + /-(pp65) | - /-(pp65) | - | + | Andro inhibits p65 phosphorylation and p65,pp65,p50 translocation into nucleus. |
| *p65-DNA binding activity* | - | ++ | + | - | - | + | |
| *TNFα, IL-1β, IkBα, IL-8* | - | ++ | + | - | - | + | |
| *Sp concentration* | 0 | ++ | + | - | + | - | Vancomycin is an antibiotic. |
| *WBC* | - | ++ | + | - | - | - | |
| *Clinical scores* | 0 | 15 | + | + | + | + | |

3.2.2 Andro dose-independently inhibited NFkB p65 phosphorylation and translocation into the nucleus. p65-DNA binding activity was reduced. Meningitis inflammation was alleviated. Sp was killed.

Table 5 Possible result 2 of each factor of rats in each group

| | Wt, sp-, DMSO | Wt, sp+, DMSO | Wt, sp+, andro+ | Wt, sp+, andro++ | Wt, sp+, ALLN, BAY | Wt, sp+, Vancomycin | |
|---|---|---|---|---|---|---|---|
| *IKK, IkB* | - | ++ | ++ | ++ | +++ | + | |
| *p65, pp65, p50 levels (cytoplasm)* | - | ++ | ++ /-(pp65) | ++ /-(pp65) | - | + | ALLN interferes with the IkB proteolysis. BAY inhibits IkB phosphorylation. |
| *p65, pp65, p50 levels (nucleus)* | - | ++ | + /-(pp65) | + /-(pp65) | - | + | Andro inhibits p65 phosphorylation and p65, pp65, p50 translocation into nucleus. |
| *p65-DNA binding activity* | - | ++ | + | + | - | + | |
| *TNFα, IL-1β, IkBα, IL-8* | - | ++ | + | + | - | + | |
| *Sp concentration* | 0 | ++ | + | + | + | - | Vancomycin is an antibiotic. |
| *WBC* | - | ++ | + | + | - | - | |
| *Clinical scores* | 0 | 15 | + | + | + | + | |

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3.2.3 Andro can't inhibit p65 phosphorylation nor translocation but did reduce inflammation.

Table 6 Possible result 3 of each factor of rats in each group

| | Wt, sp-, DMSO | Wt, sp+, DMSO | Wt, sp+, andro+ | Wt, sp+, andro++ | Wt, sp+, ALLN, BAY | Wt, sp+, Vancomycin | |
|---|---|---|---|---|---|---|---|
| *IKK, IkB* | - | ++ | ++ | ++ | +++ | + | |
| *p65, pp65, p50 levels (cytoplasm)* | - | ++ | ++ | ++ | - | + | ALLN interferes with the IkB proteolysis. BAY inhibits IkB phosphorylation. |
| *p65, pp65, p50 levels (nucleus)* | - | ++ | ++ | ++ | - | + | Andro inhibits p65 phosphorylation and p65, pp65, p50 translocation into nucleus. |
| *p65-DNA binding activity* | - | ++ | ++ | ++ | - | + | |
| *TNFα, IL-1β, IkBα, IL-8* | - | ++ | + | - | - | + | |
| *Sp concentration* | 0 | ++ | + | - | + | - | Vancomycin is an antibiotic. |
| *WBC* | - | ++ | + | - | - | - | |
| *Clinical scores* | 0 | 15 | + | + | + | + | |

3.2.4 Andro can't inhibit p65 phosphorylation nor translocation and didn't reduce inflammation.

Table 7 Possible result 4 of each factor of rats in each group

| | Wt, sp-, DMSO | Wt, sp+, DMSO | Wt, sp+, andro+ | Wt, sp+, andro++ | Wt, sp+, ALLN, BAY | Wt, sp+, Vancomycin | |
|---|---|---|---|---|---|---|---|
| *IKK, IkB* | - | ++ | ++ | ++ | +++ | + | |
| *p65, pp65, p50 levels (cytoplasm)* | - | ++ | ++ | ++ | - | + | ALLN interferes with the IkB proteolysis. BAY inhibits IkB phosphorylation. |
| *p65, pp65, p50 levels (nucleus)* | - | ++ | ++ | ++ | - | + | Andro inhibits p65 phosphorylation and p65, pp65, p50 translocation into nucleus. |
| *p65-DNA binding activity* | - | ++ | ++ | ++ | - | + | |
| *TNFα, IL-1β, IkBα, IL-8* | - | ++ | ++ | ++ | - | + | |
| *Sp concentration* | 0 | ++ | + | + | + | - | Vancomycin is an antibiotic. |
| *WBC* | - | ++ | ++ | ++ | - | - | |
| *Clinical scores* | 0 | 15 | + | + | + | + | |

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3.2.5 Andro dose-(in)dependently inhibited NFkB p65 phosphorylation and translocation into the nucleus. p65-DNA binding activity was reduced. But meningitis inflammation wasn't alleviated. Sp was killed.

Table 8 Possible result 5 of each factor of rats in each group

| | Wt, sp-, DMSO | Wt, sp+, DMSO | Wt, sp+, andro+ | Wt, sp+, andro++ | Wt, sp+, ALLN, BAY | Wt, sp+, Vancomycin | |
|---|---|---|---|---|---|---|---|
| *IKK, IkB* | - | ++ | ++ | ++ | +++ | + | |
| *p65, pp65, p50 levels (cytoplasm)* | - | ++ | ++ /- (pp65) | ++ /-(pp65) | - | + | ALLN interferes with the IkB proteolysis. BAY inhibits IkB phosphorylation. |
| *p65, pp65, p50 levels (nucleus)* | - | ++ | + /- (pp65) | - /-(pp65) | - | + | Andro inhibits p65 phosphorylation and p65,pp65,p50 translocation into nucleus. |
| *p65-DNA binding activity* | - | ++ | + | - | - | + | |
| *TNFα, IL-1β, IkBα, IL-8* | - | ++ | + | + | - | + | |
| *Sp concentration* | 0 | ++ | + | + | + | - | Vancomycin is an antibiotic. |
| *WBC* | - | ++ | ++ | ++ | - | - | |
| *Clinical scores* | 0 | 15 | + | + | + | + | |

3.2.6 Andro dose-dependently inhibited NFkB p65 phosphorylation and translocation into the nucleus. p65-DNA binding activity was reduced. Meningitis inflammation was alleviated. Sp wasn't killed.

## 4. Discussion

### 4.1 Pre-test results

#### 4.1.1 Pre-test results 1&2

This experiment is to test whether andro can enter CSF. Result 1 means andro concentration in CSF is dose-dependent to the amount of injected andro. And obviously, andro can enter CSF. Result 2 shows that andro can hardly enter CSF, which means andro are not be able to penetrate BBB even though meningitis has already made the BBB more porous. In this case, considering changing the administration routine. For example, intranasal administration, which avoids BBB, may allow more substance to enter the cerebral region.

#### 4.1.2 Pre-test results 3

IKK is significantly higher in experimental groups means the inflammation exists. No change in p65-DNA binding activity nor IL-1β expression means the NFkB pathway is inhibited by ALLN and BAY. No significant difference between wt rats and p100(-/-) rats means no compensation effect of alternative NFkB pathway has been made for the loss of the canonical NFkB pathway. So, in later inhibitory experiments, no attention needs to be paid on the alternative NFkB pathway.

#### 4.1.3 Pre-test results 4

IKK is significantly higher in experimental groups means the inflammation exists. p65-DNA binding activity of control, wt, and p100(-/-) groups are the same means the p65 canonical NFkB pathway is blocked. But IL-1β expression in wt group is significantly higher than p100(-/-) group means p100

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and alternative NFkB pathway contribute to inflammation. We can not neglect the alternative NFkB pathway. Therefore all the following experiments should be done with p100(-/-) rats.

## 4.2 Results

### 4.2.1 Result 1

This result fully supports the hypothesis. Andro dose-dependently alleviated S.p meningitis by inhibiting the canonical NFkB pathway p65/p50 subunits. Andro also killed S.p.

The blank control wt, sp-, DMSO don't have meningitis and infection. Therefore that group got all negative results and 0 clinical scores. Wt, sp+, DMSO is the negative control group. This group is Sp infected with the treatment of DMSO. The high levels indicate the meningitis model was formed.

The low concentration andro group has the same p65, pp65, p50 (cytoplasm) levels with the negative control group. But the p65, pp65, p50 (nucleus) levels are lower. This proves that andro inhibited the translocation of p65, pp65, p50 into the nucleus. As a consequence, the p65-DNA binding activity was lower than negative control because fewer p65 enters the nucleus. Then the TNFα, IL-1β, IkBα，IL-8 expression was lower because the NFkB p65 promoter was no longer bound to DNA. So the meningitis inflammation was reduced, the WBC level was lower. The sp concentration was also lower means the andro kills sp in CSF. If we look at p65 and pp65 levels separately, we can find that the pp65 level is less than the negative control group, because of the andro inhibition of phosphorylation of p65.

High concentration andro group have the same trend with higher significance. Indicating the dose-dependent character of andro.

The wt, sp+, ALLN, BAY had higher levels of IKK, IkB because they block the phosphorylation and proteolysis of IkB. Therefore the IkB and IKK accumulated. But due to the inhibition, all downstream activities were blocked. However, the sp concentration was not reduced because ALLN and BAY have no anti-bacterial function. Because the inflammation was reduced, the WBC level was lower.

In the Vancomycin group: inflammation was not reduced, but the infection was. That also caused a drop in WBC level and sp concentration.

All treatment reduced clinical scores.

In this result, we know that: (1) Andro is dose-dependent; (2) andro can inhibit NFkB pathway by blocking p65 and p50 and therefore reduce inflammation; (3) andro can kill sp.; (4) Andro performed excellently in both (2) and (3).

### 4.2.2 Result 2

This result partially supports the hypothesis. Andro dose-independently alleviated S.p meningitis by inhibiting the canonical NFkB pathway p65/p50 subunits. Andro also killed S.p.

The situation is similar to the result 1 with only one difference: more andro injected didn't improve its performance. That might because only a little dose of andro is enough for andro to do its inhibitory work. That means andro is a powerful inhibitor. Or because andro triggered some other pathways. But andro is not an excellent drug for bacterial meningitis.

### 4.2.3 Result 3

This result partially supports the hypothesis. The andro can alleviate S.p meningitis, but not by inhibiting the NFkB pathway p65/p50 subunits. And andro can eliminate S.p.

The high level of pp65 and p65 in the nucleus and low level of inflammation cytokines suggest that Andro couldn’t inhibit p65 phosphorylation nor translocation but did reduce inflammation. That’s a conflicting result with previous researches. If all the experiments were done correctly, this might suggest that sp triggers a different NFkB pathway or even an entirely different inflammation pathway. Or the meninges and brain cells have a different mechanism for regulating inflammation.

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#### 4.2.4 Result 4

This result doesn't support the hypothesis. Andro is unable to inhibit the NFkB pathway p65/p50 subunits. And it can't alleviate S.p meningitis.

This result is also a conflict with previous researches. Andro wasn't been able to show inhibitory function in CSF even though it was detectable. Maybe some enzymes or substances bind to andro, preventing it from functioning.

#### 4.2.5 Result 5

This result partially supports the hypothesis. The andro can inhibit the NFkB pathway p65/p50 subunits. And andro can eliminate S.p. But andro fails to alleviate the S.p meningitis.

p65, pp65, p50 levels, and p65-DNA binding activity level are reduced, indicating successful inhibition by andro. But the inflammation still exists. Furthermore, the ALLN, and BAY group showed that inhibiting NFkB will reduce inflammation. The compensation effect of the alternative NFkB pathway is eliminated in the pre-test. It could be inferred that the p50 has an unknown compensation effect, though existing researches believe that p50 lacks a transcriptional transactivation region and therefore can't activate inflammation cytokine expression.

#### 4.2.6 Result 6

This result partially supports the hypothesis. Andro dose-dependently alleviated S.p meningitis by inhibiting the canonical NFkB pathway p65/p50 subunits. Although, andro can't eliminate S.p.

The most situation is the same as result 1. But andro is not qualified enough to kill sp. We might consider a combined treatment: use andro and antibiotic at the same time, to improve the therapeutic efficacy.

## 5. Conclusion

This study explored the new field andrographolide can be used. The proposal designed different ways to test the competence of andrographolide to inhibit the canonical NFkB pathway and killing bacteria in vivo. p100(-/-) rats are used to eliminate the compensation effect. A new bacteria concentration measuring method is tried using GFP Fluorescent *S. pneumoniae* in CSF for the first time. It might be an impactful new drug for bacterial meningitis because of the good qualities andrographolide owns.

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