



Self-carrier nanodrugs with dual antibacterial agents to overcome tobramycin resistance in multidrug-resistant *Staphylococcus aureus*

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ABSTRACT

Multidrug-resistant (MDR) bacterial infections have emerged as a significant threat to global public health, with antibiotic resistance mechanisms—such as alterations in bacterial membrane permeability—substantially limiting therapeutic options and exacerbating infection persistence. In this study, we developed a novel self-carrier nanodrug formulation by directly assembling antibiotic tobramycin (Tob) and antimicrobial borneol 4-formylbenzoate (BF) into Tob-BF nanodrugs (TBN) via dynamic Schiff base linkages, without the need for additional adjuvants. The resultant TBN exhibits minimal bactericidal activity under physiological conditions but undergoes decomposition and activation specifically within the microenvironment of bacterial infections. Confocal microscopy analysis demonstrates that TBN is preferentially taken up and accumulates intracellularly in multidrug-resistant *Staphylococcus aureus* (MDRSA) to a greater extent than either Tob or BF alone. In both *in vitro* and *in vivo* assays, TBN outperforms Tob, BF, and physically mixed Tob + BF (with equal dose to TBN) in terms of antibacterial efficacy, particularly in promoting the recovery of xenograft infection with MDRSA in mice. Collectively, these findings highlight the potential of our self-carrier nanodrug platform, which integrates two distinct antibacterial agents, as an innovative and effective strategy to overcome tobramycin resistance in MDRSA and eliminate MDRSA infections.

1. Introduction

Multidrug resistant (MDR) bacterial infection is one of the most urgent global public healthcare concerns [1–3]. In case of infection with MDR pathogens, therapy may involve prolonged duration of last resort antibiotics at elevated doses, or, in the most resistant infections, therapy options may be severely, if not totally restricted [4,5]. The development and spread of antibiotic resistance have been aided by the over-use and misuse of antibiotics [6–8]. One of the most important mechanisms of antibiotic resistance is the decreased permeability of bacterial membrane [9–11]. Although alternatives to antibiotics are required and continuously being investigated [12], the existing armamentarium of antibiotics may still have value against MDR pathogens, if we administer them in a more effective and safe manner that bypasses these resistance

mechanisms [13].

Nanocarrier-based drug delivery systems (DDS), typically biomaterial-based polymers formulated to carry the active agent, are one approach to improve the therapeutic efficacy of active drugs while avoiding related toxic and side effects [9,14–16]. However, many of the nanocarrier-based DDS described to date have certain drawbacks, such as potential systemic toxicity of the carrier material used to deliver the active agent [17,18], and limited drug loading and releasing capacity [19]. To overcome many of these issues, nano-sized drugs fabricated from a combination of active agents without or with minimum use of inert carrier materials have been described in the treatment of tumors and termed carrier-free nanodrugs [20,21]. The carrier-free nanodrugs often combine one targeting agent and one active agent, but may also be composed of two active agents. Since there is no biomaterial component

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used in constructing these particles, they are termed “nanodrugs” rather than nanoparticles. Carrier-free nanodrugs have the following potential virtues [19,20,22,23]: (i) simple preparation; (ii) synergistic combinations of active agents; (iii) avoidance of carrier-induced toxicity; (iv) high drug loading capacity (up to 100 %). These properties of carrier-free nanodrugs, make them a particularly efficient and potent application mode for synergistic combinations of antibacterials.

To date, a small number of carrier-free nanodrugs have been developed and tested for MDR bacterial infections [24,25]. It is important to rationally design carrier-free nanodrugs based on potent combinations of antibacterial agents and consideration of molecular structure to enable assembly of nanodrugs. Finally, a better understanding of nanodrugs disassembly, and triggering this only in the infection microenvironment would be of substantial benefit in the effectiveness of the nanodrugs.

To address the above-mentioned issues, we developed a smart carrier-free nanodrug delivery system to overcome antibiotic resistance and eliminate clinical MDR bacterial infections (Fig. 1). Hydrophilic and amino group-containing tobramycin (Tob, commonly used clinical broad-spectrum aminoglycoside antibiotic) was combined with the hydrophobic aldehyde group-containing borneol 4-formylbenzoate (BF, synthesized antibacterial molecule) to directly assemble into Tob-BF nanodrugs (TBN, see Fig. 1A). The two components assemble *via* dynamic Schiff base linkages. The resulting TBN, composed of active Tob and BF themselves, enabled delivery of both antibacterial agents without relying on any carriers, but possessed carrier features due to functional groups of Tob on TBN surface, thus described as a self-carrier nanodrug. Based on the pH responsive nature of the dynamic Schiff base linkages [26], the TBN could maintain stable drug delivery nanostructures that displayed minimal bactericidal activity under normal physiological conditions, but decompose in the acidic microenvironments of bacterial infections (low pH values as far as pH 5.5) and kill MDR *Staphylococcus aureus* (MDRSA, as the model bacteria). This means that the TBN can simulate the response process of human fibrinogen for hemostasis (fibrinogen: inactive in blood under physiological conditions; but cleaved by thrombin during bleeding and converted into fibrin monomer to achieve hemostatic effect) [27]. Furthermore, uptake and intracellular accumulation of TBN increased in MDRSA, exhibiting the greatest bactericidal effect on MDRSA *in vitro* and *in vivo* compared to

control groups, including mixtures of Tob and BF (with equal dose to TBN). Overall, the self-carrier strategy for bactericides not only provides an effective method for the design of potent drug assemblies, but also robustly overcomes tobramycin resistance and achieves highly effective antibacterial activity against MDRSA infections, holding great promise for clinical applications.

2. Results

2.1. Preparation and characterization of BF and TBN

The self-carrier nanodrugs take advantage of dynamic Schiff base linkages, which maintains the antibacterial functions of Tob and BF, as well as guarantees the integrity of the nanodrug. First, borneol 4-formylbenzoate (BF) was synthesized by esterification of borneol with 4-formylbenzoic acid (Fig. S1) [28], confirmed by Fourier-transform infrared (FTIR) and proton nuclear magnetic resonance (^1H NMR) spectra (Fig. S2 and Fig. S3). Subsequently, the hydrophilic Tob molecules were fixed at the dichloromethane/water emulsion interface by hydrophobic BF molecules, producing the self-carrier TBN particles with hydrophilic surface (Fig. 1A). As shown in scanning electron microscopy (SEM) and transmission electron microscope (TEM) images (Fig. 2A and B), TBN appeared as spherical structures with high monodispersity. Subsequently, energy dispersive spectroscopy (EDS) elemental mapping demonstrated the uniform distribution of C, O, and N elements within TBN (Fig. S4). Based on dynamic light scattering (DLS) analysis (Fig. 2C), the average hydrodynamic size of TBN is 188.7 ± 7.2 nm. It had high charge density with a zeta potential of 25.13 ± 0.76 mV (Fig. 2D). Furthermore, the stability of TBN was tested in diverse media including water (H_2O), phosphate buffered saline (PBS), and tryptone soya broth (TSB). As shown in Fig. 2E, TBN kept a relatively consistent diameter in the three solvents over 7 days at 4 °C.

The Schiff base linkages of TBN were then confirmed by ultraviolet (UV)-Vis spectra. As depicted in Fig. 2F, TBN exhibited the characteristic absorption peaks of Schiff base linkages at around 270 nm, indicating the successful combination of Tob and BF. Further evidence was provided by ^1H NMR spectra (Fig. 2G). The signal peak at δ 10.1 ppm disappeared due to the reaction of the aldehyde group of BF with the amino groups of Tob. Meanwhile, the prominent signal peaks of the Schiff base

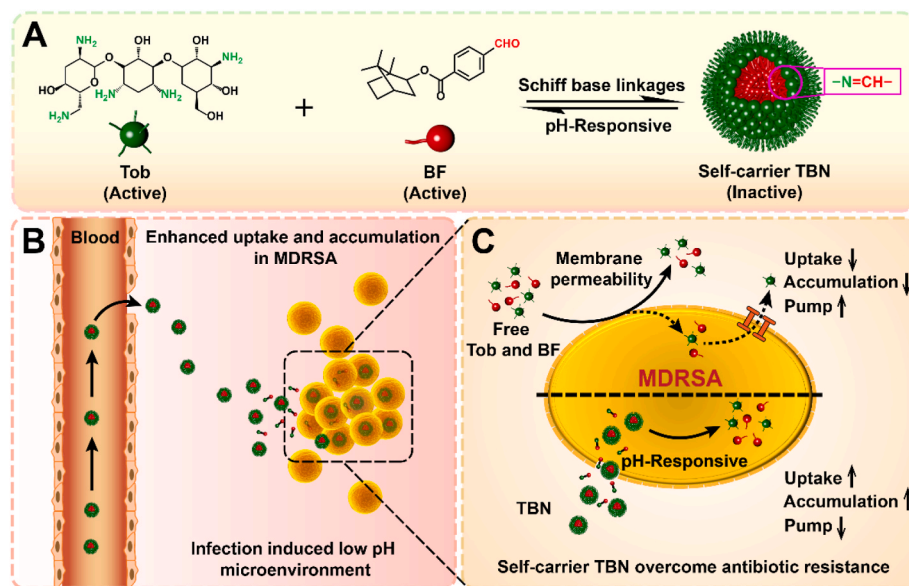


Fig. 1. Schematic illustration of the design of self-carrier Tob-BF nanodrugs (TBN) for overcoming tobramycin resistance in multidrug-resistant *Staphylococcus aureus* (MDRSA). (A) The preparation of self-carrier TBN without additional adjuvants. (B) TBN maintain stable drug nanostructures and resting antibacterial activity in blood, but decompose in the acidic microenvironments of MDR bacterial infections while enhancing uptake and intracellular accumulation in MDRSA. (C) TBN are ingested by MDR bacteria, thus overcoming antibiotic resistance and eliminating MDR bacterial infections.

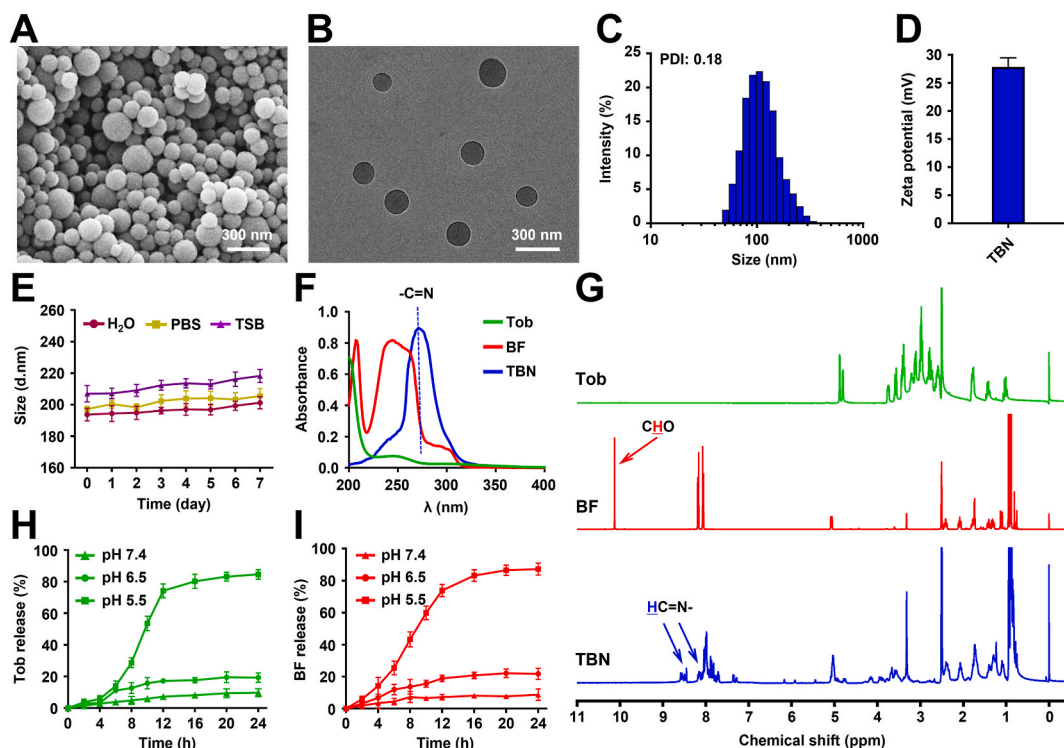


Fig. 2. Characterization of TBN. (A) SEM image, (B) TEM image, (C) particle size distribution by DLS, and (D) zeta potential of TBN ($n = 3$). (E) Stability assay of TBN in diverse media ($n = 3$). (F) UV–Vis spectra and (G) ^1H NMR of Tob, BF and TBN. *In vitro* cumulative drug-release profiles of (H) Tob and (I) BF from TBN at various pHs ($n = 3$).

linkages in TBN were found at δ 8.05–8.20 and δ 8.43–8.60 ppm, resulting in the signal shift of benzene ring (δ 7.68–8.05 ppm) on BF (Fig. S3, 5 and 6). These findings confirmed the successful combination of Tob with BF via the Schiff base linkages to form TBN. The integration ratio of representative characteristic peaks further proved that the molar ratio of Tob and BF within the TBN structure is close to 1:2 (Fig. S6).

The drug loading content (DLC) and drug encapsulation efficiency (DEE) were then determined [29]. DLC^{Tob} and DLC^{BF} reached up to $44.5 \pm 0.9\%$ and $55.9 \pm 0.8\%$ respectively (Table S1), which means that the molar ratio of the two components (Tob:BF) in TBN is 1:2, which was consistent with the molar ratio in the ^1H NMR analysis. DEE^{Tob} was $7.4 \pm 0.2\%$ and DEE^{BF} was $99.8 \pm 1.5\%$ (Table S1), which is caused by the feed ratio of preparation.

Drug release of Tob and BF were then investigated in PBS at pH 5.5, 6.5, and 7.4, which were used for physiological and increasingly acidic conditions. As shown in Fig. 2H, approximately 74.2 % of Tob was released within 12 h at pH 5.5, which was 10.3-fold higher than that at pH 7.4 ($\sim 7.2\%$) and 4.3-fold higher than that at pH 6.5 ($\sim 17.2\%$). Subsequently, a relatively steady and sustained release of Tob ($\sim 84.5\%$) lasted until 24 h of incubation at pH 5.5. However, the release of Tob was only $\sim 9.7\%$ and $\sim 19.3\%$ under physiological conditions (pH 7.4) and slight acidic conditions (pH 6.5) within 24 h, respectively. Similarly, the cumulative release of BF over 24 h was $\sim 87.2\%$ (Fig. 2I) at pH 5.5, which was about 10.0 times higher than that at pH 7.4 ($\sim 8.7\%$) and 4.0 times higher than that at pH 6.5 ($\sim 21.8\%$). These results confirmed the degradation of the TBN at lower pH, with release of Tob and BF under conditions mimicking the site of infection (low pH).

To further confirm the responsive degradation of TBN at pH 5.5, the size and morphology of TBN was evaluated. As shown in Fig. S7, the size of self-carrier nanodrug gradually increases over time, which was ascribed to the acid-labile cleavage of Schiff base linkages in TBN, leading to the hydrophobic aggregation of BF molecules in the solution. TEM also proved that TBN gradually disintegrated after 4 h, and the nanodrug completely lost its original structure after 24 h (Fig. S8). These

observations confirm that TBN demonstrates pH responsiveness in terms of drug release, size and morphology.

2.2. *In vitro* antibacterial activity

To evaluate antibacterial activity *in vitro*, the minimum inhibitory concentration (MIC) of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN was determined for log phase bacteria, using antibiotic-sensitive *Staphylococcus aureus* (*S. aureus*). As shown in Fig. S9 and Fig. S10, the MIC values of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN were 0.5 $\mu\text{g/mL}$, 2.0 $\mu\text{g/mL}$, 1.0 $\mu\text{g/mL}$ (containing 0.44 $\mu\text{g/mL}$ of Tob and 0.56 $\mu\text{g/mL}$ of BF) and 0.5 $\mu\text{g/mL}$ (containing 0.22 $\mu\text{g/mL}$ of Tob and 0.28 $\mu\text{g/mL}$ of BF), respectively.

The synergistic effect was then investigated between Tob and BF. The two ingredients, both in TBN and physically mixed Tob + BF (with equal dose to TBN), were then tested using checkerboard assays and the fractional inhibitory concentration index (FICI) [30]. The FICI of Tob and BF in the physical mixture (containing 0.44 $\mu\text{g/mL}$ of Tob and 0.56 $\mu\text{g/mL}$ of BF) was 1.10 for *S. aureus* (Fig. S11 and Table S2), indicating that there was no interaction between Tob and BF. When Tob and BF were assembled into TBN, the MIC value was decreased to 0.5 $\mu\text{g/mL}$ (containing 0.22 $\mu\text{g/mL}$ of Tob and 0.28 $\mu\text{g/mL}$ of BF), and the FICI was significantly reduced to 0.55 (Fig. S11 and Table S2), demonstrating that there is an additive effect between the two compounds [31]. To further assess bactericidal effect *in vitro*, the minimum bactericidal concentration (MBC) was also determined. The MBC of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN against *S. aureus* was 2.0 $\mu\text{g/mL}$, 4.0 $\mu\text{g/mL}$, 4.0 $\mu\text{g/mL}$, and 2.0 $\mu\text{g/mL}$, respectively (Fig. S12 and Table S2).

Furthermore, *in vitro* pH-responsive antibacterial effect was examined in real time against *S. aureus* cultures. During coinocubation of *S. aureus* with Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN, the optical density (OD600) values, colony plate images, total colony count, and pH changes of the *S. aureus* culture were

monitored over 12 h (Fig. 3A–D). *S. aureus* in control conditions experienced exponential growth within 8 h of incubation as revealed by OD600 and total colony counts. The number of *S. aureus* remained relatively stationary thereafter, till 12 h. The start of the bacterial growth phase was delayed by the treatment with both free Tob ($1.06 \times \text{MIC}$, $0.53 \mu\text{g/mL}$, the effective concentration of Tob in TBN) and free BF ($0.34 \times \text{MIC}$, $0.67 \mu\text{g/mL}$, the effective concentration of BF in TBN), with the bacteria starting to grow only after 4 h (Fig. 3B and C), suggesting that Tob or BF itself has a certain inhibitory effect on bacterial activity, but cannot completely eradicate *S. aureus* in this experimental setting (Fig. 3A–C). The physically mixed Tob + BF at an equal dose to TBN group (containing $1.06 \times \text{MIC}$ Tob, $0.53 \mu\text{g/mL}$, and $0.34 \times \text{MIC}$ BF, $0.67 \mu\text{g/mL}$) also significantly inhibited and delayed bacterial growth, but the bacteria also began to grow after 4 h (Fig. 3B and C). In comparison, when *S. aureus* was exposed to TBN ($1.20 \mu\text{g/mL}$, containing $1.06 \times \text{MIC}$ Tob, $0.53 \mu\text{g/mL}$, and $0.34 \times \text{MIC}$ BF, $0.67 \mu\text{g/mL}$; $<\text{MBC}$), a similar bacterial proliferation to control group was observed within 5 h, which indicated that TBN showed little to no inhibitory effect on *S. aureus* growth within the tested 5 h of period. After that, the CFUs in the TBN group decreased gradually and achieved complete eradication of all bacteria after 11 h of treatment (Fig. 3B and C). The increase in activity of TBN coincides with a timepoint where the pH of the culture medium drops ($\text{pH} \sim 5.2$, Fig. 3D). These results demonstrate that TBN can respond to the acidic bacterial microenvironment and eradicate the bacteria, whereas Tob, BF, and physically mixed Tob + BF (with equal dose to TBN) lack this feature and this contributed to failure to achieve complete eradication of the bacteria.

All the above test results revealed that the self-carrier TBN display little to no bactericidal activity under physiological conditions, but display pH responsive bactericidal activity in the acidic microenvironment of bacteria. This infection-site responsiveness mode endows TBN with a response process, mimicking that of human fibrinogen for hemostasis [27].

Additionally, live/dead dual-color fluorescent staining and SEM analysis were employed to evaluate the bactericidal activities of TBN [32]. Notably, exclusively dead bacteria (red fluorescence, Fig. S13A) were observed in the group treated with TBN ($2.0 \mu\text{g/mL}$, containing $1.78 \times \text{MIC}$ Tob, $0.89 \mu\text{g/mL}$, and $0.56 \times \text{MIC}$ BF, $1.12 \mu\text{g/mL}$). In contrast, after treatment with the mixture of Tob + BF (with equal dose to TBN, containing $1.78 \times \text{MIC}$ Tob, $0.89 \mu\text{g/mL}$, and $0.56 \times \text{MIC}$ BF, $1.12 \mu\text{g/mL}$), the number of dead bacteria (red fluorescence) slightly decreased. Meanwhile, when treated with Tob ($1.78 \times \text{MIC}$, $0.89 \mu\text{g/mL}$) and BF ($0.56 \times \text{MIC}$, $1.12 \mu\text{g/mL}$) alone, most of the *S. aureus* strains survived (green fluorescence). SEM was also performed to further confirm the bactericidal activities of TBN (Fig. S13B). TBN ($2.0 \mu\text{g/mL}$, containing $1.78 \times \text{MIC}$ Tob, $0.89 \mu\text{g/mL}$, and $0.56 \times \text{MIC}$ BF, $1.12 \mu\text{g/mL}$) caused bacteria to completely lose their structural integrity implying that TBN exerts a strong bactericidal ability by destroying the bacterial membrane. This reflects the results of the plate counting method shown in Fig. 3B and C. In contrast, when co-incubated with physically mixed Tob + BF (with equal dose to TBN, containing $1.78 \times \text{MIC}$ Tob, $0.89 \mu\text{g/mL}$, and $0.56 \times \text{MIC}$ BF, $1.12 \mu\text{g/mL}$), the surfaces of bacteria became rough and wrinkled and the intracellular contents were partially exuded. The bacteria treated by Tob or BF groups had normal shapes and retained clear borders and membrane integrity compared with control group. This is likely due to the expected antibacterial mechanism of Tob, which is achieved by inhibiting the translation of mRNA into proteins, rather than by destroying the cell membrane directly [33]. These results suggest that antibacterial activity is enhanced when the constitutive Tob and BF are arranged in the TBN, rather than given in a simple mixture. This highlights the benefit of the TBN structure over its constitutive parts.

To prove that TBN can overcome antibiotic resistance and combat MDR bacteria, we repeated these experiments by testing activity towards a clinical MDR *S. aureus* (MDRSA). The MIC of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN against MDRSA were

$16.0 \mu\text{g/mL}$, $8.0 \mu\text{g/mL}$, $8.0 \mu\text{g/mL}$ (containing $3.56 \mu\text{g/mL}$ of Tob and $4.47 \mu\text{g/mL}$ of BF), and $2.0 \mu\text{g/mL}$ (containing $0.89 \mu\text{g/mL}$ of Tob and $1.12 \mu\text{g/mL}$ of BF), respectively (Fig. 3G, Fig. S14 and Fig. S15). As revealed in Fig. 3E, only partial inhibition of bacterial growth was observed in MDRSA when treated with Tob alone, the Tob could not eradicate MDRSA completely even at a concentration up to $256.0 \mu\text{g/mL}$ ($16 \times \text{MIC}$), which proved that this MDRSA is resistant to Tob. Conversely, BF ($2 \times \text{MIC}$, $16.0 \mu\text{g/mL}$), physically mixed Tob + BF (with equal dose to TBN, $2 \times \text{MIC}$ BF, $16.0 \mu\text{g/mL}$, the concentration shown is the BF concentration), and TBN ($1 \times \text{MIC}$ BF, $8.0 \mu\text{g/mL}$, the concentration shown is the BF concentration) could completely kill all MDRSA.

We further assessed the synergy of the Tob and BF against MDRSA, and found the fractional inhibitory concentrations (FICs) of Tob and BF were significantly reduced in combination compared to those observed for *S. aureus*. When Tob and BF were used as the physically mixed Tob + BF with an MIC of $8.0 \mu\text{g/mL}$ (containing $0.22 \times \text{MIC}$ Tob, $3.56 \mu\text{g/mL}$, and $0.56 \times \text{MIC}$ BF, $4.47 \mu\text{g/mL}$), the FICI value was 0.78, showing an additive effect against MDRSA. Interestingly, when Tob and BF were assembled into TBN, the MIC of TBN was reduced to $2.0 \mu\text{g/mL}$ (containing $0.06 \times \text{MIC}$ Tob, $0.89 \mu\text{g/mL}$, and $0.14 \times \text{MIC}$ BF, $1.12 \mu\text{g/mL}$), having a FICI value of 0.19, which demonstrated synergy between Tob and BF in the nanoparticle assembly (Fig. 3F and G). Thus, the TBN assembly significantly promoted synergy against MDRSA compared with the simple mixture of constitutive components. These results suggested that TBN displays superior inhibition of the MDRSA growth and eradication of bacteria than physically mixed Tob + BF (with equal dose to TBN).

Subsequently, we also performed a live/dead staining assay and SEM observation to further characterize the antibacterial effect against MDRSA [32]. MDRSA treated with TBN ($1 \times \text{MIC}$ BF, $8.0 \mu\text{g/mL}$, the concentration shown is the BF concentration) almost all died (red fluorescence, Fig. S16A). In contrast, after treatment with the BF ($1 \times \text{MIC}$, $8.0 \mu\text{g/mL}$) and physically mixed Tob + BF (with equal dose to TBN, $1 \times \text{MIC}$ BF, $8.0 \mu\text{g/mL}$, the concentration shown is the BF concentration), partial inhibition of bacterial growth and a small amount of dead MDRSA strains (red fluorescence) were observed. Whilst the Tob ($0.4 \times \text{MIC}$, $6.4 \mu\text{g/mL}$) treated MDRSA emitted intense green fluorescence. Bacterial morphology was also observed by SEM to further evaluate the bactericidal activities of TBN. The SEM images (Fig. S16B) showed that the TBN caused substantial damage to the bacterial membrane with leakage of contents. However, the MDRSA treated by BF ($1 \times \text{MIC}$, $8.0 \mu\text{g/mL}$) and physically mixed Tob + BF (with equal dose to TBN, $1 \times \text{MIC}$ BF, $8.0 \mu\text{g/mL}$, the concentration shown is the BF concentration) were slightly deformed, and leakage of contents was noted sporadically. Additionally, the MDRSA in Tob treatment group retained clear borders and membrane integrity similar to the control group (Fig. S16B). These results indicated that synergistic antibacterial behaviors of TBN could overcome the resistance of MDRSA to Tob and promote TBN to effectively eradicate MDRSA.

Based on these data, we hypothesized that the TBN formulation could increase uptake by MDRSA and lead to greater intracellular accumulation, thus overcoming antibiotic resistance and yielding highest synergistic antibacterial effect (FICI was 0.19) compared to Tob, BF, and physically mixed Tob + BF (with equal dose to TBN) groups. This would then allow us to further verify the mechanistic action of TBN.

2.3. Mechanisms of action of TBN

One of key reasons many antibiotics fail to treat MDR bacteria is that they cannot be efficiently internalized by these bacteria [34,35]. To verify the internalization of TBN by MDRSA, the colocalization of TBN with MDRSA was performed by confocal laser scanning microscope (CLSM) [36]. First, Nile red (NR)-loaded TBN (denoted as NR-TBN, red) were co-incubated for 3 h with MDRSA to investigate TBN internalization, and MDRSA were marked in green fluorescence by lectin from *Triticum vulgare* (wheat) FITC conjugate (FITC-WGA) [37]. CLSM images

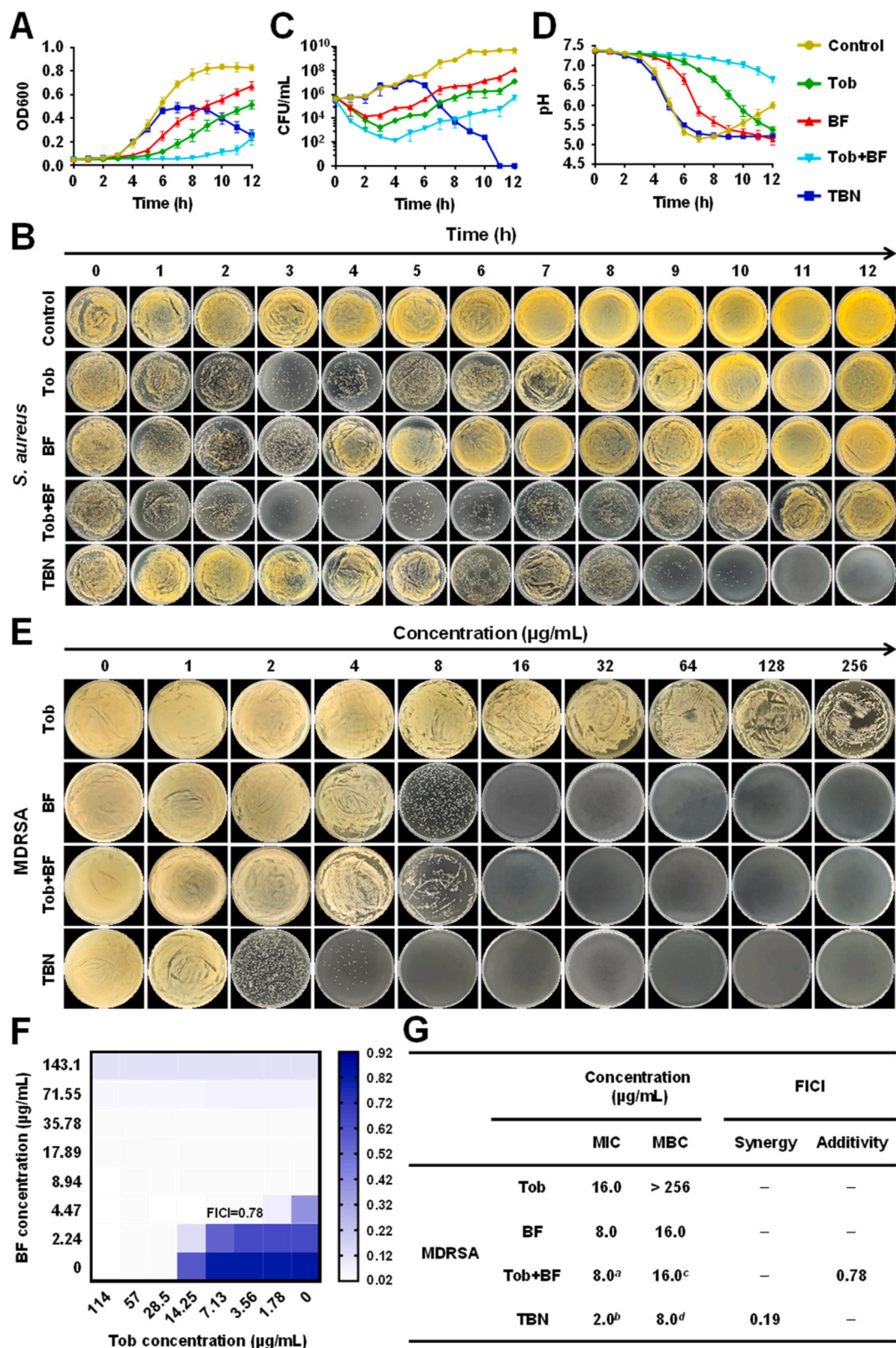


Fig. 3. *In vitro* antibacterial assessment of TBN. Time course of activity revealed by (A) optical density (OD600), (B) quantitative bacteriological culture *via* plate images, and (C) *via* total colony count, and (D) pH of culture media in control, Tob (1.06 × MIC, 0.53 µg/mL), BF (0.34 × MIC, 0.67 µg/mL), physically mixed Tob + BF (with equal dose to TBN, containing 1.06 × MIC Tob, 0.53 µg/mL, and 0.34 × MIC BF, 0.67 µg/mL), and TBN (1.20 µg/mL, containing 1.06 × MIC Tob, 0.53 µg/mL, and 0.34 × MIC BF, 0.67 µg/mL) groups over 12 h (n = 3). (E) Photos of MDRSA treated with different concentrations of Tob, BF, physically mixed Tob + BF (with equal dose to TBN, in terms of BF), and TBN. (F) Checkerboard assays performed on MDRSA to evaluate the synergy between Tob and BF using the fractional inhibitory concentration index (FICI). (G) MIC, MBC, and FICI of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN against MDRSA. ^a3.56/4.47 (Tob/BF), ^b0.89/1.12 (Tob/BF), respectively. For ^cphysically mixed Tob + BF and ^dTBN, the concentration shown is the BF concentration.

show that a considerable number of NR-TBN are internalized by MDRSA cells, as strong red fluorescence is observed inside the bacterial cells from two overlapped channels (Fig. 4A). In contrast, no red fluorescence signal is observed inside MDRSA cells treated with free NR, which is highly co-localized with FITC-WGA cell membrane staining. The significant difference in cellular uptake between NR and NR-TBN groups proved that the TBN can be efficiently internalized by MDRSA.

Additionally, to further monitor the internalization process of TBN over time, we employed coumarin 6-loaded TBN (denoted as Cou 6-TBN, green) and co-incubated them with MDRSA stained by NR (Fig. S17) [38]. After 1 and 3 h of co-incubation, MDRSA treated with Cou 6-TBN showed strong green fluorescence signals in bacterial cells, and the signal from two channels overlapped well (Fig. S17B). Nevertheless, a rare green fluorescence signal of free hydrophobic Cou 6 was distributed in MDRSA, demonstrating a lower intracellular uptake of Cou 6 by MDRSA within same 3 h of treatment (Fig. S17A). After further co-incubation for 6 h, weaker green fluorescence signals were observed inside MDRSA cells that treated with Cou 6-TBN, because many MDRSA cells were killed by Cou 6-TBN due to pH-responsive bactericidal effect. Meanwhile, no substantial Cou 6 signal was found within MDRSA cells until incubation times over 6 h. The significant difference in cellular uptake between free Cou 6 and Cou 6-TBN groups further proved that the TBN could be internalized by MDRSA over time.

MDRSA utilize the proton motive force (PMF) across their cytoplasmic membranes to generate ATP. PMF disruption can impair ATP synthesis, thereby reducing the active transport of drugs and diminishing bactericidal efficacy [39]. Preincubation of MDRSA with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore that dissipates PMF, inhibits the uptake of aminoglycosides and enhances bacterial survival [40]. To explore the internalization mechanism of TBN, MDRSA were preincubated with CCCP before exposure to TBN. The results displayed that CCCP pretreatment significantly increased bacterial colony counts compared to untreated MDRSA in a TSB nutrient environment (Fig. 4B, ****, $p < 0.0001$), indicating that PMF inhibition reduced the bacterial uptake of TBN and diminished its antibacterial

activity. Conversely, CCCP did not significantly affect the bactericidal efficacy of TBN under saline conditions, underscoring the importance of PMF in mediating TBN's effects (Fig. 4C, ns, $p = 0.056$). Therefore, we hypothesize that TBN is efficiently taken up by MDRSA via PMF-facilitated active transport, exerting its antibacterial effects intracellularly.

We hypothesized that the TBN could effectively enhance bacterial cell membrane permeability of MDRSA, which might make the diffusion of TBN into the cells more easily. The internalized TBN could further induce leakage of intracellular contents, subsequently damaging the bacterial membranes and leading to bacterial cell death. To verify this hypothesis, TBN was co-incubated with SYTO 9-labeled MDRSA (green) [41] for 0, 1, 3, and 6 h respectively to validate the effect of TBN on bacterial cell membrane permeability. After 1 and 3 h of TBN treatment, the green fluorescence intensity of SYTO 9-labeled MDRSA was essentially consistent with that of the control group at 0 h (Fig. S18). This is because TBN possess the biomimetic response mechanism, maintaining stable drug self-delivery nanostructures and displaying minimal antibacterial activity for MDRSA. However, the green fluorescence in the TBN group was significantly decreased after co-incubation for 6 h, indicating that TBN enhanced the permeability of the SYTO 9-labeled MDRSA cell membranes, leading to the leakage of fluorescent substances from within the bacteria.

The surface membrane potential is closely associated with both the permeability and structural integrity of bacterial cell membranes [42]. Therefore, the temporal changes in membrane potential of MDRSA were measured following treatment with TBN. As illustrated in Fig. S19, the membrane potential of MDRSA significantly increased after 3 and 6 h of TBN treatment compared to 0 h (*, $p = 0.0199$ at 3 h, **, $p = 0.0031$ at 6 h), which attributable to the positive surface charge of TBN (+25.13 mV). Previous study demonstrated that cationic interactions with anionic sites on bacterial surfaces can disrupt bacterial physiological states [43]. The positive charge on TBN surface facilitates its binding to MDRSA, thereby disturbing the membrane potential and enhancing the bacterial cell membrane permeability.

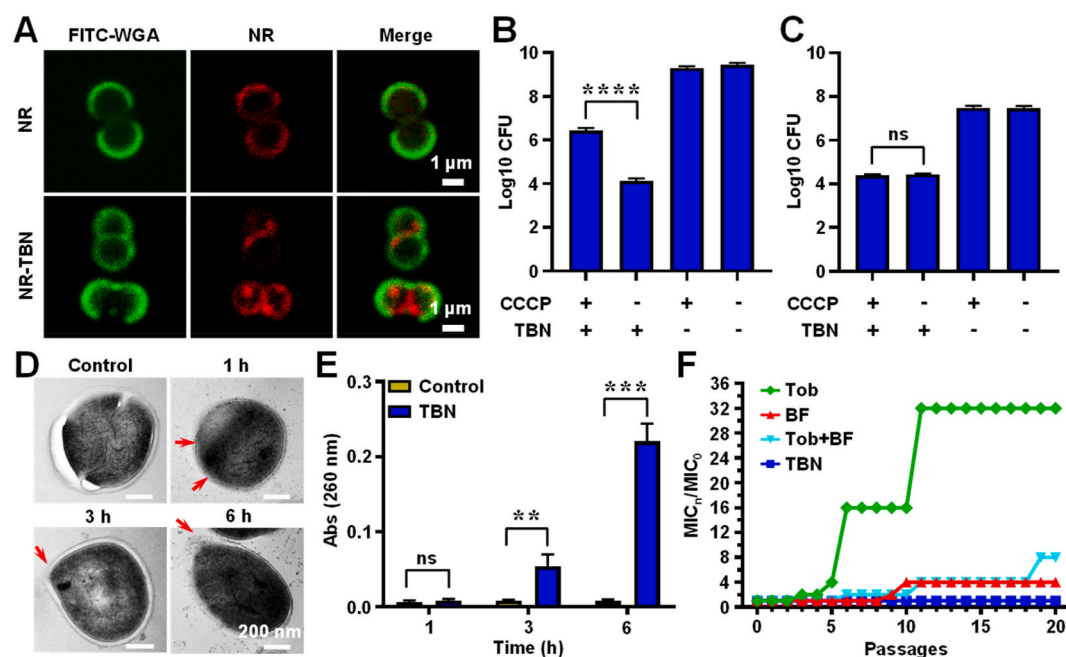


Fig. 4. Mechanisms of overcoming bacterial resistance by TBN. (A) CLSM images of MDRSA after incubation with NR and NR-TBN for 3 h at 37 °C. (B) Colony counts of MDRSA after co-incubation with TBN under the influence of CCCP in (B) TSB environment and (C) NaCl environment ($n = 3$). **** $p < 0.0001$ and ns $p > 0.05$. (D) In situ bio-TEM images of MDRSA after incubation with TBN for 1, 3, and 6 h at 37 °C, respectively. The red arrows denote TBN attaching to the MDRSA surface. (E) The release of intracellular contents identified via absorption at 260 nm from MDRSA by treatment with TBN ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, and ns $p > 0.05$ respectively. (F) Drug resistance development profiles of *S. aureus* during serial passaging in the presence of sub-MIC dosing of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN. The y-axis indicates the folds of increased MIC compared to the initial ones.

Subsequently, the morphological changes of MDRSA following TBN treatment at different time points were observed using SEM and in situ bio-TEM. Compared with the clear and smooth boundary exhibited by the bacteria in control group, SEM images (Fig. S20) confirmed that there was no significant change in MDRSA after treatment with TBN for 1 and 3 h. After 6 h of treatment, membrane disruption, bacterial collapse, and leakage of intracellular contents were observed in the bacteria. It was also demonstrated by bio-TEM images (Fig. 4D), showing a complete cellular morphology of the MDRSA in control group

and treated with TBN at 1 and 3 h, respectively. After TBN treatment for 6 h, the interaction between TBN and bacterial cells caused damage to the bacterial membranes (red arrows), suggesting that TBN has the potential to gradually induce the leakage of intracellular contents and disrupt membrane integrity by enhancing the permeability of bacterial cell membranes.

Next, we quantitatively evaluated the leakage of intracellular contents from MDRSA caused by TBN. The increase of absorbance at 260 nm was an indication of the intracellular contents released from bacterial

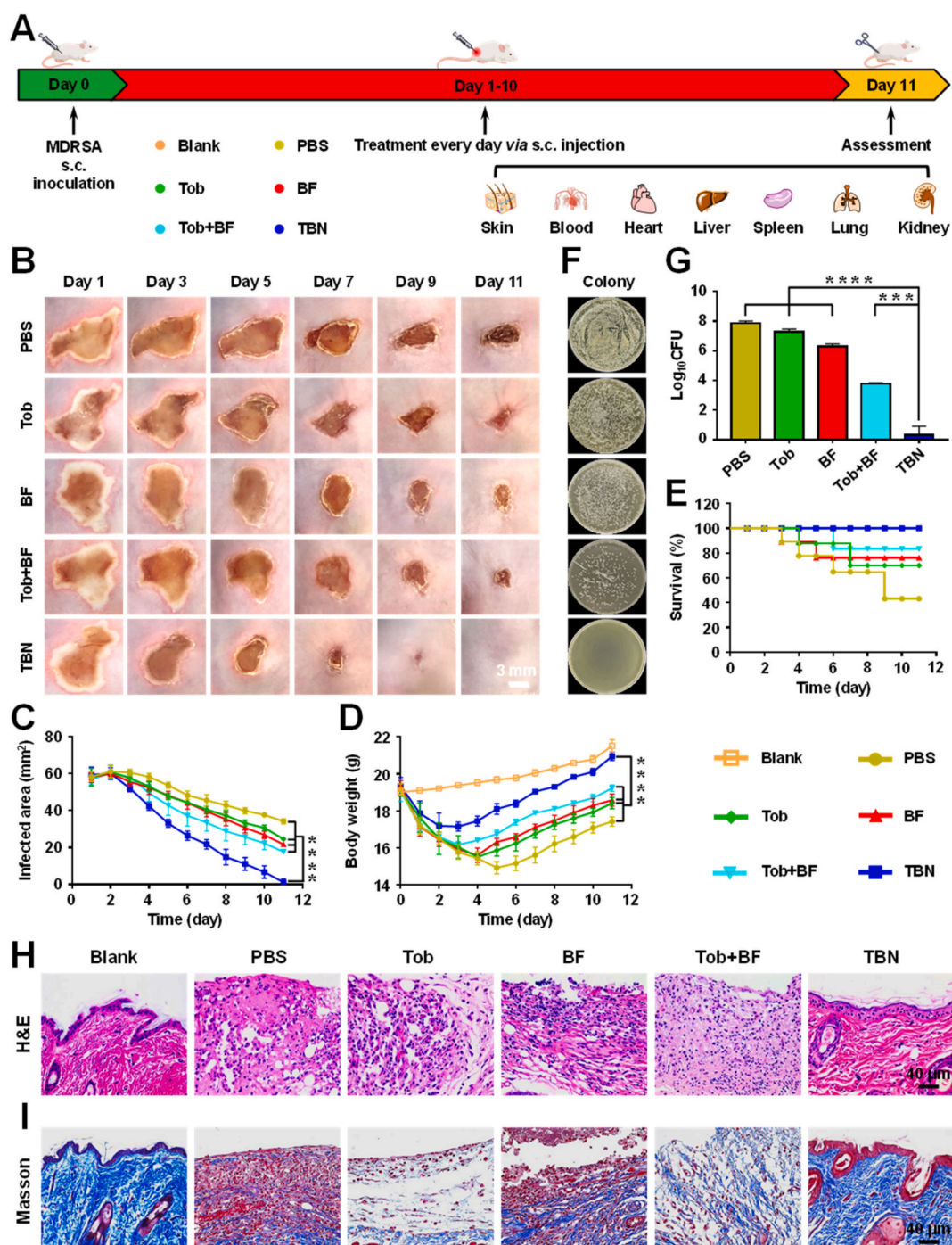


Fig. 5. *In vivo* antibacterial activity of TBN. (A) Schematic diagram of the experimental process. (B) Representative photographs of the MDRSA-infected mice during treatment with the different formulations for 10 days (n = 6). (C) The size of the infected areas (n = 6). (D) The body weight of the infected mice (n = 6). (E) Percentage survival of infected mice during treatment. Photographs of plated bacterial colonies (F) and quantitative results (G) of bacterial cultures obtained from infected skin tissues of mice in the five treatment groups (n = 3). (H) H&E staining and (I) Masson's trichrome staining (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, compared with TBN respectively. All experiments were repeated three times, with n = 6 mice per group in each independent experiment.

cells [44]. As shown in Fig. 4E, the release of bacterial intracellular contents was time-dependent. Compared with the control group, the release of the intracellular substances was significantly enhanced with TBN treatment (16.0 $\mu\text{g/mL}$, containing $0.45 \times \text{MIC}$ Tob, 7.12 $\mu\text{g/mL}$, and $1.12 \times \text{MIC}$ BF, 8.94 $\mu\text{g/mL}$) as the co-incubation duration increased (ns, $p = 0.059$ at 1 h, **, $p = 0.0078$ at 3 h and ***, $p = 0.0001$ at 6 h). The leakage of intracellular contents (e.g. nucleic acids) that absorb at a wavelength of 260 nm indicates disruption and damage to the bacterial cytoplasmic membrane [44]. Therefore, TBN could enhance the permeability of cell membranes, causing the leakage of intracellular components and damage to bacterial cell membranes.

Overall, the experimental evidence presented above highlights the advantages of the integration of Tob with BF into TBN for improving antibacterial activity against MDRSA. On one hand, TBN can be effectively internalized by MDRSA. On the other hand, TBN can enhance the permeability of MDRSA cell membranes, thereby inducing the leakage of intracellular components and causing membrane damage. This may facilitate the entry of more antimicrobial agents and TBN into MDRSA, thereby further enhancing intracellular accumulation [45]. TBN then responds to release Tob and BF, which increase local drug concentration surrounding and within MDRSA, thereby exerting an efficient synergistic bactericidal effect to overcome tobramycin resistance.

2.4. Prevention of resistance development

The repeated use of antibiotics will promote the selection of drug resistant pathogens and lead to antimicrobial resistance [6]. To investigate the propensity of TBN toward resistance development, susceptible *S. aureus* were treated for multiple generations with a sub-lethal dose of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN ($0.5 \times \text{MIC}$) for 20 passages [46]. As illustrated in Fig. 4F and Fig. S21, free Tob rapidly induced strong drug resistance, and led to a 32-fold increase in MIC after 20 passages, indicative of the development of Tob-resistance. In comparison, free BF and physically mixed Tob + BF (with equal dose to TBN) caused a significant reduction and a delayed resistance, yielding 4 times and 8 times increase in MIC, respectively. But, there was no change of MIC of TBN against *S. aureus* over 20 passages.

2.5. In vivo eradication of skin infection

Encouraged by the excellent antibacterial performance *in vitro*, we further explored the *in vivo* antibacterial efficacy using MDRSA-induced mouse skin infection model [47]. As shown in Fig. 5A, each mouse was inoculated with MDRSA (1×10^8 CFU/mL, 100 μL) by subcutaneous (s.c.) injection. After 24 h observation, the inoculated mice were treated with PBS, free Tob ($0.89 \times \text{MIC}$, 14.24 $\mu\text{g/mL}$), free BF ($2.24 \times \text{MIC}$, 17.89 $\mu\text{g/mL}$), physically mixed Tob + BF (with equal dose to TBN, containing 14.24 $\mu\text{g/mL}$ of Tob and 17.89 $\mu\text{g/mL}$ of BF), and TBN (32.0 $\mu\text{g/mL}$, containing 14.24 $\mu\text{g/mL}$ of Tob and 17.89 $\mu\text{g/mL}$ of BF) *via* in situ s.c. injection, respectively. Fig. 5B shows representative photographs of MDRSA infected mice over 10 days of treatment. The bacterial infected skin observed in the four control groups, PBS, Tob, BF, and physically mixed Tob + BF (with equal dose to TBN), displayed significant scars or ulcerations after 10 days of treatment. Conversely, almost a full recovery of lesions was observed in infected mice treated by TBN at the therapy endpoint (infected area: 1.17 mm^2), which was significantly lower than that of PBS (34.13 mm^2 ; ***, $p < 0.0001$), Tob (24.55 mm^2 ; ***, $p < 0.0001$), BF (21.65 mm^2 ; ***, $p < 0.0001$), and physically mixed Tob + BF (with equal dose to TBN, 17.63 mm^2 ; ***, $p < 0.0001$) treated mice, indicating that TBN had better antibacterial efficiency than the other groups (Fig. 5C). Meanwhile, the body weight of the infected mice in TBN group increased fastest than all other treatment groups (***, $p < 0.0001$, Fig. 5D). The survival rate of mice was 43 % with PBS after 10 days of treatment, whereas the survival rate of mice was remarkably increased after treatment with Tob (70 %), BF (76 %),

and physically mixed Tob + BF (with equal dose to TBN, 83 %). Notably, at the end of the monitoring period, treatment with TBN led to higher survival rate (100 %) than that of other groups (Fig. 5E).

The bactericidal effect was further validated by excising the infected tissues and quantifying the number of bacteria on TSA plates. As demonstrated in Fig. 5F, the CFUs of surviving MDRSA of TBN ($0.33 \log_{10}$ CFU) were significantly lower than that of PBS ($7.87 \log_{10}$ CFU; ***, $p = 0.000025$), Tob ($7.29 \log_{10}$ CFU; ***, $p = 0.000038$), BF ($6.30 \log_{10}$ CFU; ***, $p = 0.000068$), and physically mixed Tob + BF (with equal dose to TBN, $3.77 \log_{10}$ CFU; ***, $p = 0.0005$), confirming that the TBN exhibited the highest MDRSA antibacterial efficacy *in vivo* (Fig. 5G). This suggests that TBN could effectively eliminate MDRSA, as our *in vitro* data indicate that this was achieved by increased uptake of MDRSA and enhanced intracellular accumulation of antibacterials acting in synergy.

Furthermore, hematoxylin and eosin (H&E) staining (Fig. 5H) was performed on skin tissue sections to examine the recovery of the infected skin tissues [48]. Compared to uninfected skin tissue in the blank group, significantly higher levels of inflammatory cells, severely disrupted dermis structures and infected skin lesions were found in the sections from group treated with PBS. After treatment with Tob, BF, and physically mixed Tob + BF (with equal dose to TBN) respectively, although the inflammation was relieved in each mouse, the infected tissues still displayed varying degrees of neutrophil accumulation, fibrous tissue proliferation, and focal necrosis. In comparison, the skin tissues obtained from TBN group exhibited relatively intact histological characteristics with more blood vessels, thickened epidermis, and well fibroblast, demonstrating the best outcomes were observed for TBN treated animals. Additionally, the Masson's trichrome staining assay was used to verify the formation of collagen fibers (blue) during skin infection healing [49]. Unrepaired collagen fibers were observed in the infected tissues after the four control treatments, whereas more newly produced and well-established collagen fibers were deposited in the infected tissues treated with the TBN (Fig. 5I), which suggesting that TBN treatment had favorable effects on the recovery of skin infection. This view was also supported by inflammatory response evaluation in skin tissues and blood by the enzyme-linked immunosorbent assay (ELISA, Fig. S22). The expression of inflammatory markers, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) [50], of skin tissues and blood in TBN group was dramatically lower than those in PBS (**, $p < 0.01$), Tob (**, $p < 0.01$), BF (**, $p < 0.01$), and physically mixed Tob + BF (with equal dose to TBN, *, $p < 0.05$, corresponded to the expression of TNF- α in skin tissues; **, $p < 0.01$, corresponded to the expression of TNF- α in blood). These results demonstrated that an obvious reduction in inflammatory response of TBN treatment during infection recovery, which further confirmed that the TBN played a positive role in the treatment of MDRSA induced skin infection.

During recovery and tissue regeneration, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) are two main growth factors that regulate cell proliferation, differentiation, and migration [51]. Thus, the infected tissues collected from different treatment groups were double immunofluorescent stained with VEGF and EGF to assess the healing of the skin (Fig. 6A). A large number of positively stained VEGF (red) and EGF (green) cells appeared in TBN treated tissues at day 10, which are essential for infected skin recovery and tissue repair. Conversely, little secretion of VEGF and EGF growth factors was observed in other groups. Semi-quantitative analysis (Fig. 6C and D) exhibited that TBN significantly upregulated the VEGF (66.89) and EGF (53.14) expression compared with PBS (VEGF: 25.04, EGF: 15.93; ***, $p < 0.0001$), Tob (VEGF: 45.05, EGF: 28.77; ***, $p < 0.0001$), BF (VEGF: 45.99, EGF: 30.58; ***, $p < 0.0001$), and physically mixed Tob + BF (with equal dose to TBN, VEGF: 57.33, ***, $p < 0.001$; EGF: 42.42, **, $p < 0.01$) groups. During the tissue repair process, the higher expression of EGF in the TBN group strongly contributed to keratinocyte migration and fibroblast proliferation, thus promoting

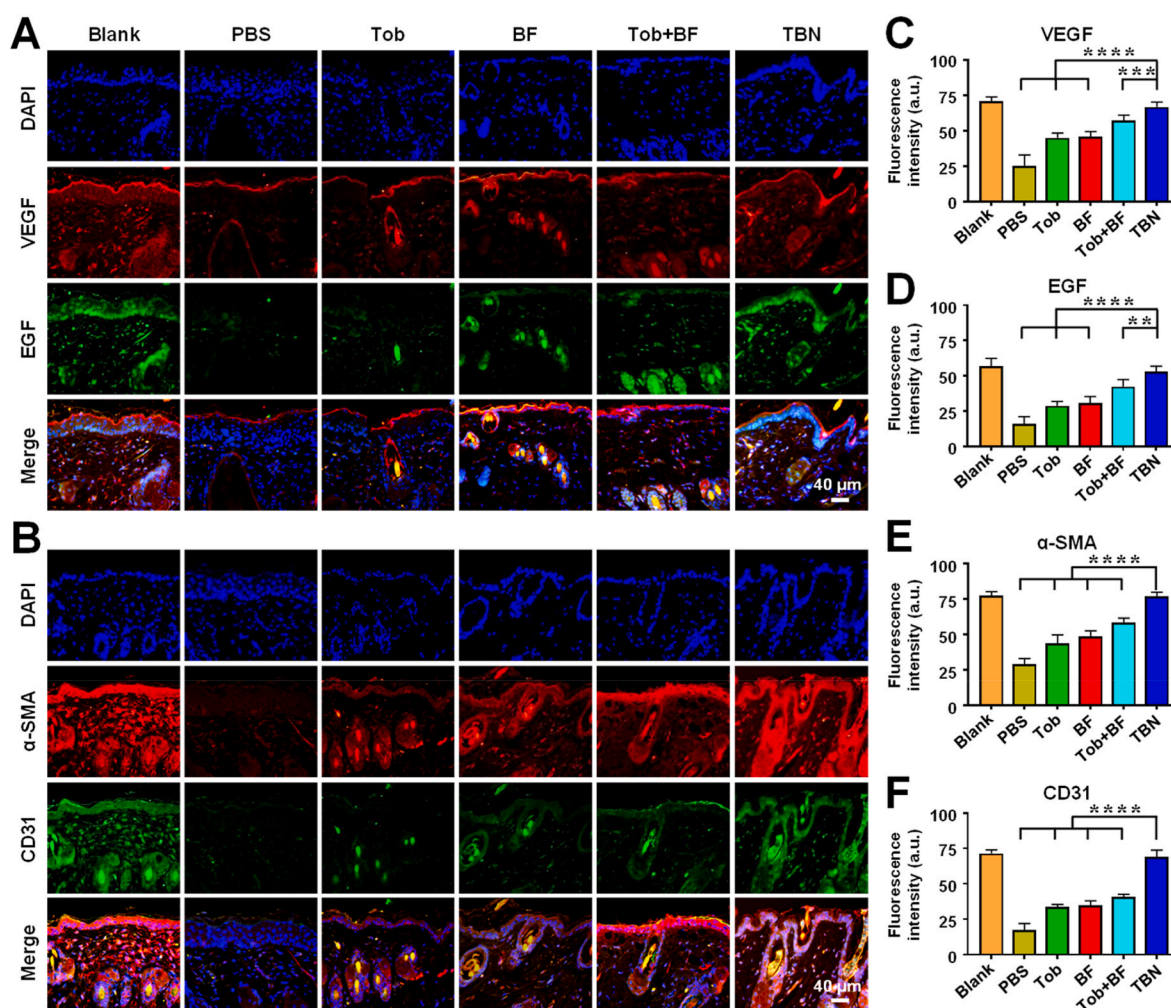


Fig. 6. Infected skin tissues recovery after 10 days treatments by immunofluorescence co-labeling staining. (A) VEGF (red) and EGF (green), (B) α -SMA (red) and CD31 (green). The cell nuclei were stained blue. Semi-quantitative analysis of fluorescence intensity in sections stained for (C) VEGF, (D) EGF, (E) α -SMA, and (F) CD31, respectively ($n = 6$). $**p < 0.01$, $***p < 0.001$, compared with TBN respectively.

infected skin healing and re-epithelialization [52]. Additionally, the more secretion of VEGF in TBN-treated tissue also plays an important role in regulating angiogenesis, re-epithelization, and collagen deposition [53].

Angiogenesis is essential for skin tissue development and maturation. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a vascular endothelial cell marker and mainly reflected the newly-formed blood vessels [54]. Meanwhile, alpha smooth muscle actin (α -SMA) can mark the vascular smooth muscle cells. The formation of mature vasculature is characterized by the endothelial cells being surrounded by smooth muscle cells [55]. Thus, the immunofluorescence co-staining of α -SMA (red) and CD31 (green) can be used to evaluate neo-vascularization and mature vessels in infected sites with different treatments [56]. As showed in Fig. 6B, these blood vessels were densely distributed in the TBN group, whereas few positive cells of CD31 and α -SMA was detected in the PBS, Tob, and BF groups. In the physically mixed Tob + BF (with equal dose to TBN) group, the vascular structures were present at a moderate level. Quantitation fluorescence intensity of α -SMA (76.98) and CD31 (68.86) in the infected tissues treated with TBN indicated that the production of vascular structures (Fig. 6E and F).

Taken together, the self-carrier TBN could eliminate MDRSA efficiently *in vivo* and further accelerate infected skin recovery and tissue regeneration. This can be attributed to the beneficial effects of the biomimetic fibrinogen-responsive-like property and synergistic antibacterial activity of TBN. Under the stimulation of the acidic environment in

the infected sites, both Tob and BF can be released from TBN in a controllable manner to combat MDRSA. Specifically, TBN could increase MDRSA uptake and intracellular accumulation effectively (Fig. 4A and Fig. S17), which further realized the synergistic sterilization inside the bacteria. Conversely, Tob, BF, and physically mixed Tob + BF (with equal dose to TBN) had an unsatisfactory effect due to the lack of biomimetic response process and failure to induce synergistic antibacterial properties.

2.6. *In vivo* biomimetic responsive antibacterial evaluation

In vivo distribution of TBN was assessed. Mice received intravenous (i.v.) injection with Cyanine 7.5-loaded TBN (denoted as Cy7.5-TBN), and were monitored with an *in vivo* imaging system (IVIS). Cy7.5 signals were observed in the liver in 1 h and enhanced after 3 h of treatment, whereas very weak signals were observed in other organs (Fig. S23A).

To evaluate the *in vivo* targeting property of TBN, a skin infection model was used to determine the *in vivo* real-time distribution of TBN [57]. As shown in Fig. S23B, PBS (100 μ L) and MDRSA (1×10^8 CFU/mL, 100 μ L) were injected subcutaneously into the left back and right back of the mice (namely uninfected and infected sites), respectively. After 24 h treatment, Cy7.5-TBN were administrated *via* i.v. injection and imaged by IVIS. Evident fluorescence signals were observed mainly distributing around the liver and the infected site at 6 h

post-injection due to the enhanced permeability and retention (EPR) effect. Compared to the uninfected site, the fluorescence signal of the infected site showed a gradual increase over time and finally reached the strongest at 24 h.

The *in vivo* biomimetic responsive antibacterial efficiency of TBN was verified by employing two additional infection models. First, the skin infection model with MDRSA (1×10^8 CFU/mL, 100 μ L) was constructed and treated with PBS and TBN (10 mg/kg) *via* i.v. injection at 24 h post-inoculation (Fig. 7A). Compared with the PBS group, the infected area of mice in TBN group gradually decreased with increasing time, which achieved almost full recovery after 12 days treatment (Fig. 7B). This result implied that the TBN could target MDRSA-induced skin infection sites, and responsively decompose and release bactericides activated by the acidic microenvironment of bacterial infections, thus showing rapid and incomparable synergistic sterilization.

To further investigate the biomimetic responsive antibacterial mechanism of TBN, healthy mice were administered PBS and TBN (10 mg/kg) *via* i.v. injection, respectively. After 24 h treatment, all mice were then inoculated (s.c.) with MDRSA (1×10^8 CFU/mL, 100 μ L, Fig. 7C). As presented by Fig. 7D, the mice in TBN group exhibited slight skin infection on day 1. As time went on, the infected areas gradually decreased until disappeared completely at day 4, whereas the infected site of the mice treated with PBS was still obvious visible. These results further demonstrated that the TBN can maintain stable self-carrier nanostructure and resting bactericidal activity under physiological conditions *in vivo*. Once attacked by MDRSA, TBN can effectively prevent the occurrence of skin infection, exhibiting outstanding antibacterial capability toward MDRSA. Based on these results, we proposed that self-carrier TBN is a superior antibacterial tool for eradicating MDRSA-induced skin infection synergistically and preventing infection

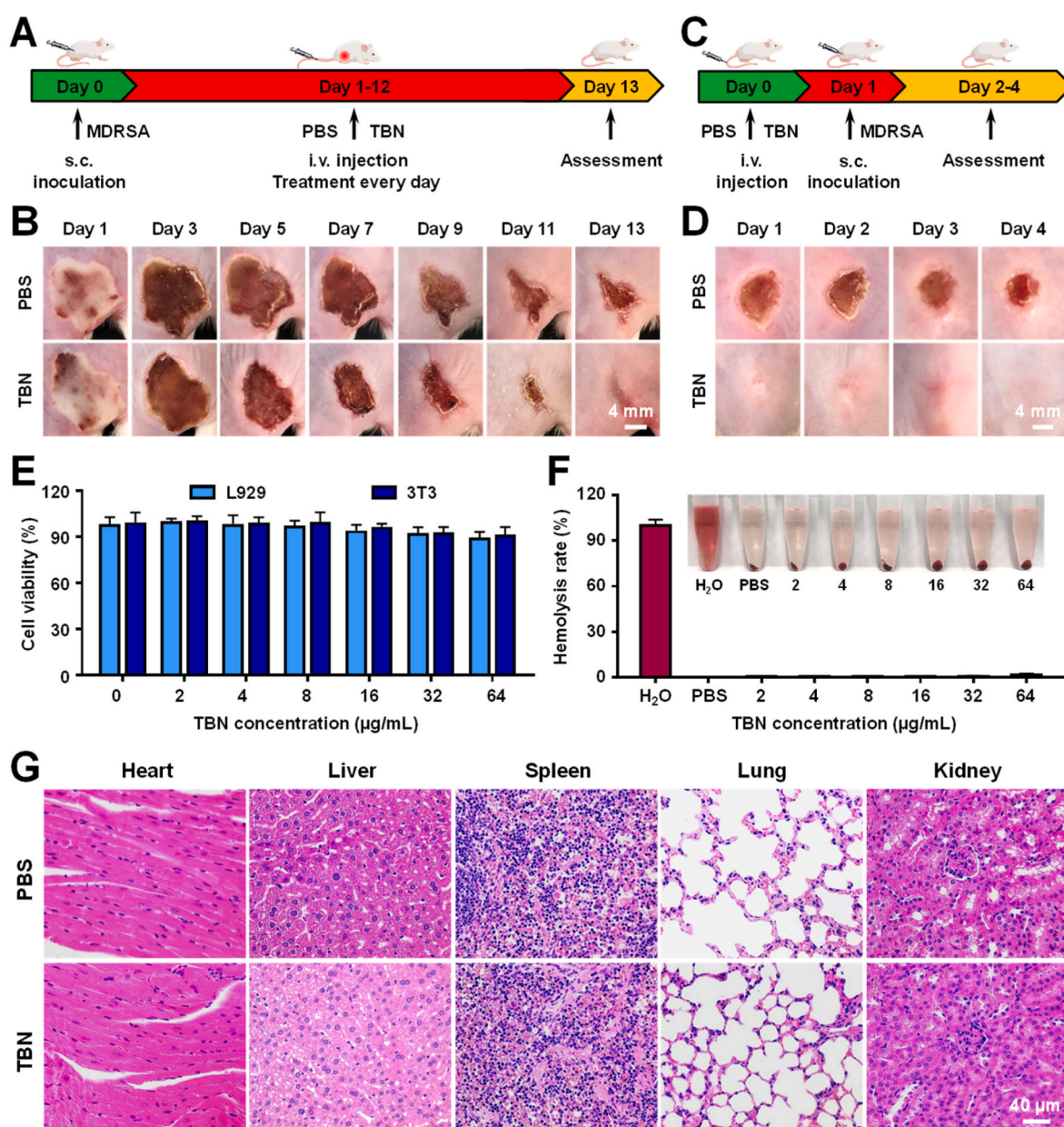


Fig. 7. *In vivo* biomimetic response and safety evaluation. (A) Schematic diagram of the antibacterial experimental process through tail vein injection. (B) Representative photographs of the MDRSA-infected mice during treatment with PBS and TBN for 10 days ($n = 3$). (C) Schematic diagram of the experimental process of biomimetic response. (D) Representative photographs of the mice after MDRSA infection for 4 days ($n = 3$). (E) Cell viability of L929 and MC3T3-E1 cells treated with different dosages of TBN for 24 h, respectively ($n = 6$). (F) Hemolysis evaluation of TBN with different concentrations ($n = 4$). (G) *In vivo* H&E-stained tissue sections of major organs (heart, liver, spleen, lung, and kidney) in mice 12 days after the tail vein injection of TBN.

effectively through the biomimetic response process of human fibrinogen *in vivo*.

2.7. *In vitro* and *in vivo* biocompatibility evaluation

For therapeutic use of antibacterial materials, it is necessary to possess significant activity against bacteria while displaying low toxicity and great systemic safety [58]. The cytotoxicity of TBN was first tested at the cellular level. After incubation of TBN with the mouse fibroblast cells (L929) and mouse embryonic osteoblasts cells (MC3T3-E1) for 24 h, the cytotoxicity was evaluated by standard MTT assay [59]. As shown in Fig. 7E, the TBN had no obvious cytotoxicity on the two cells, the cell viability remained more than 89 % in the concentration range from 2 to 64 $\mu\text{g/mL}$, indicating that TBN had good biocompatibility at the cellular level. Additionally, cytotoxicity of Tob and BF in TBN was also evaluated using L929 cells and MC3T3-E1 cells. After 24 h of incubation with Tob and BF at concentrations ranging from 2 to 64 $\mu\text{g/mL}$, both cell types exhibited high viability ($\geq 90\%$, Fig. S24), demonstrating the excellent biocompatibility of Tob and BF. Furthermore, the hemolytic activity of TBN was examined against red blood cells [60]. The hemolysis induced by TBN was 1.47 % at a concentration up to the 64 $\mu\text{g/mL}$ (Fig. 7F), implying no obvious erythrocyte damage was observed.

In vivo blood biochemical analysis in serum was performed to quantitatively evaluate the influence of TBN on healthy mice [61]. The major indexes of liver and kidney functions in TBN group, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CRE), total bilirubin (TBIL) and total protein (TP), showed no significant statistical differences compared to the PBS control group (Fig. S25), demonstrating that the TBN had no significant toxicity to the treated mice. Besides, H&E staining of the representative organs (heart, liver, spleen, lung, and kidney) was also performed to prove long-term biosafety of TBN. As revealed in Fig. 7G, the tissues of TBN group maintained normal structures without noticeable signs of organ damage or histopathological lesions compared to PBS controls. All the above results collectively suggested the excellent biosafety of TBN as an effective platform to eradicate MDRSA bacterial skin infection.

3. Discussion

The overuse and misuse of antibiotics have accelerated the development of antibiotic resistance, with one important mechanism being decreased bacterial membrane permeability. However, existing antibiotics may still be effective against MDR pathogens if administered more effectively and safely. Carrier-free nanodrugs, which offer desirable advantages over nanocarrier-based DDS, provide a potent mode for synergistic combinations of antibacterials. However, studies focused on the rational design of carrier-free nanodrugs to combat bacterial infections are limited. Current antibacterial carrier-free nanodrugs are mainly assembled via non-covalent interactions [62,63], with a few employing covalent bonds [25,64].

Non-covalent interactions are pivotal in the construction of carrier-free nanodrugs for bacterial infections [62]. For instance, Li et al. assembled antimicrobial berberine (BBR) and baicalin (BA) into carrier-free BA-BBR nanoparticles (NPs) through electrostatic and hydrophobic interactions [24], which exhibited enhanced antimicrobial activity against *S. aureus* compared to free BBR. Fu et al. formulated BBR/CGA nanoparticles using BBR and chlorogenic acid (CGA) via π - π stacking and hydrogen bonding [65]. These nanoparticles demonstrated superior antibacterial activity against *S. aureus* and MRSA compared to ampicillin, BBR, CGA, and their mixture. Zheng et al. prepared carrier-free FA-BBR NPs using BBR and ferulic acid (FA) via non-covalent interactions [66], which showed superior antibacterial activity against *S. aureus* compared to BBR or FA alone. Additionally, Zhang et al. developed carrier-free RI@NP by combining rifampicin (RIF) and isopropylcarillin (IBC) via hydrophobic interactions and π - π

stacking [67]. RI@NP displayed enhanced antibacterial efficacy compared to free RIF and IBC. However, its stability and polarity required improvement with surfactants. Although carrier-free nanodrugs assembled via non-covalent interactions have demonstrated enhanced antibacterial efficacy, they may still encounter potential challenges [62,63]. The instability of non-covalent interactions may lead to premature drug release and reduced efficacy. Furthermore, these carrier-free nanodrugs generally lack environmental sensitivity, with drug release typically driven by the physical degradation of nanostructures or diffusion from particle surfaces. Additionally, research on the antibacterial efficacy of these carrier-free nanodrugs against MDR bacteria remains insufficient.

Therefore, developing stimuli-responsive carrier-free nanodrugs based on covalent bonds can control drug release, thereby enhancing drug utilization efficiency at the bacterial infection sites [63]. To date, only a limited number of such carrier-free nanodrugs have been developed for antibacterial applications, particularly against MDR bacteria. As reported in the literature, Zeng et al. assembled phenylboronic acid (PBA), sulfamethoxazole (Su), and emodin (Em) into carrier-free self-delivery nanodrugs (Su/Em/PBA) via boron ester bonds and hydrophobic interactions [64]. The Su/Em/PBA targeted bacterial infection sites and released Su and Em in response to the acidic microenvironment, exhibiting effective antibacterial activity against *S. aureus* and *E. coli*. Similarly, Liu et al. developed a carrier-free nanodrug (NEP) comprised of nitrofurantoin aldehyde (NIT), epigallocatechin-3-gallate (EGCG) and PBA through non-covalent and boronic ester bonds [25]. NEP showed enhanced antibacterial activity against *S. aureus* and *E. coli*, with concurrent anti-inflammatory activity. Notably, it remains unclear whether the synergistic antibacterial activity of carrier-free nanodrugs against MDR bacteria is superior to that of a two-drug mixture in these studies.

To enhance the stability and efficacy of carrier-free nanodrugs against MDR bacteria, strategic selection of antibacterial agents, detailed consideration of their molecular structures, and thorough understanding of nanodrug decomposition in response to the infection microenvironment are essential. Addressing these challenges, we proposed smart self-carrier TBN without additional adjuvants to reliably overcome antibiotic resistance and eliminate clinical MDR bacterial infections. The TBN was successfully assembled using the antibiotic Tob and the antibacterial agent BF via dynamic Schiff base linkages. Both Tob and BF served dual roles as antibacterial agents and carrier molecules, enabling self-loading and self-delivery. Under physiological conditions, TBN showed remarkable stability, with only $\sim 9.7\%$ of Tob and $\sim 8.7\%$ of BF released within 24 h. Furthermore, TBN exhibited minimal bactericidal activity under physiological conditions but decomposed in the acidic microenvironments of bacterial infections. Specifically, TBN displayed preferential uptake and intracellular accumulation in MDRSA. Collectively, these advantages endow TBN with superior synergistic bactericidal efficacy (FICI of 0.19) for overcoming tobramycin resistance and combating MDRSA infections, clearly superior to Tob, BF, and physically mixed Tob + BF (with equal dose to TBN).

Based on the aforementioned findings, our self-carrier nanodrugs share common advantages with reported carrier-free nanodrugs, including simple assembly, high drug loading capacity, avoidance of carrier-induced toxicity, and significant antibacterial benefits. However, several scientific issues and challenges need to draw attention for future clinical applications against MDR bacteria [18,20]: (i) Limited exploration of compatibility between antibacterial agents. Further research is needed to elucidate assembly mechanisms, optimize drug selection, and clarify synergistic effects; (ii) Insufficient targeting efficiency. Carrier-free nanodrugs often exhibit limited targeting efficiency due to restricted surface modification. An ideal carrier-free nanodrug should effectively target infected sites and penetrate barriers to ensure optimal distribution and delivery; (iii) Inadequate understanding of *in vivo* biological behavior. A comprehensive understanding of their biological behavior *in vivo* is essential before clinical application; (iv) Incomplete

biosafety investigation. Current toxicity assessments are often limited to major organs (heart, liver, spleen, lungs, and kidneys), while potential side effects and immune responses in other tissues remain underexplored; (v) Limited animal models. Given the significant differences observed when transitioning from mouse models to human preclinical trials, more human-relevant animal models are needed to rigorously verify *in vivo* efficacy and safety.

4. Conclusion

In summary, the novel self-carrier nanodrugs, TBN, are presented based on the direct assembly of Tob and BF via dynamic Schiff base linkages. Relative to other drug delivery systems, the resultant TBN achieved self-loading and self-delivery without relying on any carriers. TBN displayed high synergistic bactericidal efficacy (FICI is 0.19) for bactericides in combating MDRSA infection. Especially, TBN could exhibit minimal bactericidal activity (self-delivery) under physiological conditions, but targeted bacterial infection microenvironment and intelligently releasing antibacterial agents (acid response) for sterilization, displaying the features of mimicking the response process of fibrinogen for hemostasis. This smart TBN integrated the merits of carrier-free nanodrug, carrier features, pH-responsive, synergetic germicidal efficacy and good biosafety. In-depth analysis showed that TBN could increase bacterial uptake and intracellular accumulation in MDRSA, whilst yielding no antimicrobial resistance. These advantages endowed TBN with appealing *in vitro* and *in vivo* performances of overcoming tobramycin resistance and eliminating clinical xenograft MDRSA effectively, which distinctly outperformed the control groups of free Tob, free BF, and physically mixed Tob + BF (with equal dose to TBN). Overall, this work highlights the tremendous potential of self-carrier nanodrugs with biomimetic feature, which may provide potential opportunities for overcoming clinical antibiotic resistance and eradicating MDR bacterial infections. Firstly, given the extensive variety of antibacterial agents available in clinical practice, the strategic selection of antibacterial agent partners and a thorough consideration of their molecular structures can significantly enhance the synergistic potentiation of antibacterial activity against MDR pathogens. Secondly, enhancing the targeting capabilities of carrier-free or self-carrier nanodrugs and conducting in-depth investigations into their *in vivo* pharmacokinetics and biological transport characteristics are essential. Such research will facilitate a more profound understanding of the structure-activity relationships of these nanodrugs, which is critical for overcoming antibiotic resistance. We believe that our unique biomimetic self-carrier strategy holds the potential to rejuvenate ineffective antibiotics for the treatment of clinically antibiotic-resistant infections.

5. Materials and methods

5.1. Materials

4-Formylbenzoic acid ($\geq 97\%$), L-borneol ($\geq 99\%$), N,N'-dicyclohexyl carbodiimide (DCC, $\geq 99\%$), 4-dimethylaminopyridine (DMAP, $\geq 99\%$), coumarin 6 (Cou 6, $\geq 98\%$), Nile red (NR, $\geq 98\%$), and lectin from *Triticum vulgaris* (wheat) FITC conjugate (FITC-WGA, Protein, $\sim 50\%$ Lowry) were purchased from Sigma-Aldrich (U.S.A.). Tobramycin (Tob, $\geq 94\%$) was purchased from Tokyo Chemical Industry (TCI). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, $\geq 98\%$) were purchased from Solarbio Life Sciences (Beijing, China). Tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), and ethanol (EtOH) were purchased from J&K Scientific. Tryptone soya broth (TSB) and tryptone soy agar (TSA) were purchased from Sinopharm Chemical Reagent (China). Live/Dead® BacLight™ Bacterial Viability Kit was purchased from Thermo Fisher Scientific Inc. All other chemicals were purchased from Aladdin and used without further purification.

Cell culture media were purchased from Gibco BRL (U.S.A.) and CORNING (U.S.A.). Thiazolyl blue tetrazolium bromide (MTT) were

purchased from Solarbio Science & Technology Co., Ltd. (China). Mouse tumor necrosis factor α (TNF- α) ELISA Kit, mouse interleukin 6 (IL-6) ELISA Kit, mouse Interleukin 1 β (IL-1 β) ELISA Kit and mouse monocyte chemotactic protein 1 (MCP-1) ELISA Kit were purchased from Cusabio (China). Anti-VEGFA antibody [VG-1] (mouse), anti-EGF antibody (rabbit), anti- α -SMA antibody (mouse), anti-CD31 antibody (rabbit), TRITC goat anti-mouse IgG antibody, and FITC goat anti-rabbit IgG antibody were all purchased from Abcam (China).

5.2. Preparation of TBN

The self-carrier Tob-BF nanodrugs (TBN) were synthesized by employing interfacial Schiff-base bonding reaction. In brief, hydrophobic BF (30 mg) was dissolved in CH_2Cl_2 (1 mL). Afterward, the CH_2Cl_2 containing BF was mixed with deionized water (3.36 mL) under stirring at 3000 rpm for 6 h to obtain an emulsion template. Then, Tob solution (3 % in deionized water, 1.64 mL) was added in above system for another 1 h stirring. Subsequently, the emulsion was transferred into a dialysis bag (MWCO 1000 Da) and dialyzed against deionized water for 48 h, yielding TBN without unbound Tob.

The preparation of Nile red-loaded TBN (NR-TBN), coumarin 6-loaded TBN (Cou 6-TBN), and Cyanine 7.5-loaded TBN (Cy7.5-TBN) were following the procedure for fabricating TBN, where the hydrophobic Nile red (NR), coumarin 6 (Cou 6), or Cyanine 7.5 (Cy7.5) was added to the BF/ CH_2Cl_2 system before the interfacial Schiff base bonding reaction.

5.3. Characterization

The surface chemistry was characterized by Fourier-transform infrared (FTIR) spectra (Nicolet Antaris II, Thermo Fisher, U.S.A.). Specifically, the target borneol 4-formylbenzoate (BF) and TBN powder were each co-ground with KBr and analyzed by FTIR after pressing into tablets. The structures of BF and TBN were confirmed by proton nuclear magnetic resonance (^1H NMR) spectra recorded on a Bruker spectrometer (400 MHz, Bruker AVANCE III, Switzerland). Ultraviolet (UV)-Vis spectra were performed on a UV-Vis spectrometer (UV-2450, Shimadzu, Japan). The particle sizes and zeta potential of the TBN were detected by dynamic light scattering (DLS, ZetaSizer Nano ZS90, Malvern Instruments, U.S.A.). The morphologies of TBN and bacteria were observed using scanning electron microscopy (SEM, JSM-7800F, JEOL, Japan) and transmission electron microscopy (TEM, HT-7700, Hitachi, Japan), respectively. The elemental composition was analyzed by energy dispersive spectroscopy (EDS, JSM-7800F, JEOL, Hitachi, Japan) mapping. The absorption intensity of bacteria was measured by a microplate reader (Sunrise, Tecan Group Ltd., Zurich, Switzerland). The real-time pH changes in the co-incubation system during the biomimetic response antibacterial test were monitored by a pH meter (SevenCompact™, METTLER TOLEDO, Switzerland). Bacterial uptake was visualized using confocal laser scanning microscope (CLSM, Lecia SP8, German). Fluorescence images were collected by a fluorescence microscope (MF43-N, China).

5.4. In vitro release of Tob or BF

To evaluate the kinetics of pH-triggered Tob release *in vitro*, TBN was dispersed in PBS solution (10 mg/mL, 1 mL) with different pH (5.5, 6.5 and 7.4) respectively. Then they were added to the dialysis tube (MWCO 1000 Da), and subsequently immersed in 30 mL PBS buffer with corresponding conditions and gently shaking (180 rpm) at 37 °C. At different time intervals (from 0 h to 24 h), 1 mL of released media was retrieved and replaced with the same volume of fresh media. The absorbance at 204 nm was analyzed by UV-Vis spectrophotometer. The cumulative released Tob was quantified and carried out in triplicate with the Tob calibration curve. The amount of BF released was determined following the quantitative procedure of Tob, where PBS solution with 0.5 % Tween

80 was used as the release media [68].

5.5. Bacteria and bacteria culture

Staphylococcus aureus (*S. aureus*, ATCC 25923) was obtained from China Center of Industrial Culture Collection (China). Clinical multidrug (ampicillin, ciprofloxacin and tetracycline) resistant *Staphylococcus aureus* (MDRSA, 120016-1) was provided by Department of Microbiology of Academy of Military Medical Sciences (China). *S. aureus* and MDRSA were used as the model bacteria to evaluate the antibacterial efficiency of TBN. Bacteria were cultured in the TSB medium with a shaking (180 rpm) at 37 °C, then harvested at the logarithmic growth phase and washed twice with saline.

5.6. The measurement of MIC and MBC

To investigate the antibacterial effect of TBN, the minimum inhibitory concentration (MIC) of the different treatments on *S. aureus* and MDRSA was determined using microplate broth microdilution method with minor modifications [69]. Briefly, TSB media (100 µL) containing two-fold serial dilutions of Tob, BF, physically mixed Tob + BF (with equal dose to TBN), and TBN were placed into a 96-well plate, respectively. The bacteria were diluted to an appropriate level (1×10^6 CFU/mL). Then, the bacteria suspensions (100 µL) were added to the plate. After 24 h incubation at 37 °C, the optical density at 600 nm (OD600) was measured using a microplate reader. The MIC was determined as the lowest treatment concentration at which no visible bacterial growth compared to the control. To determine minimum bactericidal concentration (MBC), the cultures from any well of a 96-well plate (with no visible growth in the MIC assessment) were inoculated onto TSA plates and incubated for 24 h. The lowest concentration at which no single colony growth on TSA plates was evaluated as MBC [70]. MIC and MBC experiments were conducted in triplicates ($n = 3$).

5.7. FICI measurement

The combinatorial effect between Tob and BF in physically mixed Tob + BF (with equal dose to TBN), and TBN was evaluated by checkerboard assays, and the fractional inhibitory concentration index (FICI) was calculated using the following equations [30,31]:

$$FICI = MIC_{\text{combination Tob}} / MIC_{\text{alone Tob}} + MIC_{\text{combination BF}} / MIC_{\text{alone BF}}$$

The interactions between Tob and BF are defined according to standard criteria: $FICI \leq 0.5$ represented as synergy, $0.5 < FICI \leq 1$ was interpreted as additivity, $1 < FICI \leq 4$ indicated no interaction, and $FICI > 4$ showed antagonism. All experiments were performed in triplicate ($n = 3$).

5.8. In vitro pH-responsive antibacterial evaluation

The pH-response to bacterial killing kinetics of TBN was dynamically assessed by monitoring OD600, colony plate images, colony count, and pH changes during the coincubation system. The *S. aureus* strains were used as a bacterial model. Briefly, 0.5 mL of *S. aureus* suspension (1×10^7 CFU/mL) was added into 9.5 mL of TSB containing different formulations and incubated at a constant temperature (37 °C) with shaking (180 rpm) for 12 h. The final coincubation systems were control (no treatment), Tob ($1.06 \times MIC$, 0.53 µg/mL), BF ($0.34 \times MIC$, 0.67 µg/mL), physically mixed Tob + BF (with equal dose to TBN, containing $1.06 \times MIC$ Tob, 0.53 µg/mL, and $0.34 \times MIC$ BF, 0.67 µg/mL), and TBN (1.20 µg/mL, containing $1.06 \times MIC$ Tob, 0.53 µg/mL, and $0.34 \times MIC$ BF, 0.67 µg/mL; <MBC), respectively. At the defined time points (per hour), 100 µL of each culture was withdrawn for OD600 with a microplate reader. Bacterial growth curves were created and bacterial growth

kinetics were studied by plotting OD600 values versus time. Meanwhile, 100 µL of each culture and its resulting culture dilution (per hour) was dispersed onto TSA plates respectively. After 24 h incubation at 37 °C, bacterial colonies on the plates were counted on a log scale against time. To assess pH-responsive antimicrobial activity, pH changes were also determined in real-time using a pH meter. All experiments were repeated at least 3 times.

5.9. MDRSA and TBN colocalization assay

MDRSA (1×10^7 CFU/mL) was first incubated with NR-TBN (2.0 µg/mL, containing $0.06 \times MIC$ Tob, 0.89 µg/mL, and $0.14 \times MIC$ BF, 1.12 µg/mL) at 37 °C. After 3 h of treatment, MDRSA were stained with a lectin from *Triticum vularis* (wheat) FITC conjugate (FITC-WGA) at a final concentration of 20 µg/mL for 20 min and then imaged by CLSM. Furthermore, MDRSA (1×10^6 CFU/mL) was incubated with hydrophobic free Cou 6 and Cou 6-TBN (16.0 µg/mL, containing $0.45 \times MIC$ Tob, 7.12 µg/mL, and $1.12 \times MIC$ BF, 8.94 µg/mL) at a final concentration at 37 °C for 1, 3, and 6 h, respectively. Afterward, MDRSA were stained with NR (0.5 mg/mL) for 10 min and imaged with CLSM.

5.10. Influence of CCCP co-incubation

To investigate the energy-dependent internalization mechanism of TBN, MDRSA were preincubated with the carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2 µg/mL) for 10 min to dissipate transmembrane proton gradients. After centrifugation (6000 rpm, 3 min), MDRSA were resuspended in sterile TSB or isotonic saline (0.9 % NaCl) to form CCCP-treated and untreated MDRSA groups. Equimolar concentrations of TBN were then co-incubated with both CCCP-treated and untreated MDRSA at 37 °C with orbital agitation (200 rpm) for 6 h. Bacterial viability was quantitatively assessed by serial dilution in sterile PBS, followed by plating on TSA and incubation at 37 °C for 24 h. Bactericidal efficacy was determined as the log10 reduction in CFU relative to untreated controls ($n = 3$).

5.11. Morphological changes of MDRSA

Bacteria were collected and dehydrated for SEM analysis as the following method. MDRSA (1×10^6 CFU/mL) was incubated with TBN (16.0 µg/mL, containing $0.45 \times MIC$ Tob, 7.12 µg/mL, and $1.12 \times MIC$ BF, 8.94 µg/mL) at a final concentration. After 1, 3, and 6 h of treatment respectively, the bacteria were washed with saline and fixed with 2.5 % glutaraldehyde at 4 °C overnight. Then, the samples were dehydrated with sequential treatments of 30 %, 50 %, 60 %, 70 %, 80 %, 90 %, and 100 % ethanol for 10 min, respectively. Finally, the dried bacterial samples were inspected using SEM. For biological TEM samples preparation, MDRSA were collected, fixed, and stained with 0.1 % osmic acid. The samples were then dehydrated following above method. The samples were then embedded in resin, cut into ultrathin sections, stained with 2 % uranyl acetate and 0.2 % lead citrate for TEM observation. MDRSA with no treatment.

5.12. Bacterial cell membrane integrity study

The integrity of bacterial cell membranes was assessed by the leakage of intracellular components (e.g. proteins, DNA and RNA), which could be measured by using a UV-Vis spectrophotometer at 260 nm [44]. Briefly, MDRSA were diluted to 1×10^6 CFU/mL. MDRSA was incubated with TBN (16.0 µg/mL, containing $0.45 \times MIC$ Tob, 7.12 µg/mL, and $1.12 \times MIC$ BF, 8.94 µg/mL) at 37 °C for 1, 3, and 6 h, respectively. Each group was passed through a 0.22 µm filter to obtain the supernatant and measured at 260 nm using a UV-Vis spectrophotometer. A control group contained MDRSA without TBN.

5.13. Evaluation of antibiotic resistance development

The *S. aureus* was used to study the drug resistance development of free Tob, free BF, physically mixed Tob + BF (with equal dose to TBN), and TBN under the sublethal dose treatment [46]. Briefly, the bacteria (1×10^6 CFU/mL, 100 μ L) were seeded in 96 well plates and exposed to the media (100 μ L) containing a serially diluted free Tob, free BF, physically mixed Tob + BF (with equal dose to TBN), and TBN. After incubation at 37 °C for 24 h, the OD600 value in each well was measured to determine the MIC. The bacterial suspension of $0.5 \times$ MIC well was taken out at passage 0 and plated on the TSA agar plate overnight, and the MIC of these samples was determined at passage 1. The bacterial suspension of $0.5 \times$ MIC well at passage 1 was taken out for the measurement of MIC at passage 2. Similarly, MIC was measured at passage 3–20. The MIC of each passage was recorded and normalized to that of passage 0 to determine the drug resistance development.

5.14. In vitro cell biocompatibility

Cell biocompatibility of Tob, BF, and TBN were performed using the MTT assay [59]. The mouse fibroblast cells (L929) and mouse embryonic osteoblasts cells (MC3T3-E1) were seeded in 96-well plates (5000 cells per well) respectively. After 24 h incubation (37 °C, 5 % CO₂), 100 μ L of Tob, BF, and TBN at a series of concentrations (0, 2, 4, 8, 16, 32, and 64 μ g/mL) were added into the wells and incubated for another 24 h. Finally, 10 μ L of MTT (5 mg/mL) was added into each well with further incubation for 4 h at 37 °C. Then, 100 μ L 10 % SDS was added into each well for 12 h. The cell viability was determined by measuring optical density at 570 nm (OD570) with a microplate reader ($n = 6$).

5.15. In vitro hemolysis assay

Hemolysis assay was carried out on red blood cells (RBCs). Typically, the RBCs were collected from the anticoagulated blood of mice. Then, 0.2 mL of diluted RBCs were added into 0.8 mL of a series of TBN at different concentrations in PBS (2, 4, 8, 16, 32, and 64 μ g/mL). PBS (+RBCs) and H₂O (+RBCs) were chosen as negative and positive controls, respectively. These solutions were incubated in a shaker at 37 °C for 3 h. After centrifugation at 10,000 g for 5 min, the absorbance of the supernatant was measured at 540 nm using a UV–Vis spectrophotometer ($n = 4$). The hemolysis (%) was calculated by the following formula [60]:

$$\text{Hemolysis (\%)} = (\text{Abs}_{\text{Samples}} - \text{Abs}_{\text{Negative}}) / (\text{Abs}_{\text{Positive}} - \text{Abs}_{\text{Negative}}) \times 100 \%$$

Where Abs_{Samples}, Abs_{Negative} and Abs_{Positive} correspond to the absorbance of the samples, negative control and positive control, respectively.

5.16. Animals

Male Balb/c mice (6 weeks, 18–20 g) were purchased from Beijing Charles River Co., Ltd. and used to evaluate *in vivo* antibacterial activity and biosafety. All animals were treated and cared for in accordance with the National Research Council's Guidelines for the care and use of laboratory animal. All animal experiments were approved for animal ethics and welfare permission from the Animal Ethics Committee of the China-Japan Friendship Hospital (the Approval no: zryhy12-20-08-3) and carried out under the supervision and evaluation of the SPF Animal Department of Clinical Institute in China-Japan Friendship Hospital. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to obtain statistically significant data.

5.17. In vivo antibacterial activity

The MDRSA-induced skin infection model was established in each

mouse to evaluate the antibacterial efficacy of TBN. Briefly, all mice were first shaved to remove dorsal hair. Then, 100 μ L of MDRSA suspension (1×10^8 CFU/mL) was subcutaneously inoculated into the back of the mice. After infection for 24 h, MDRSA-infected mice were randomly divided into 5 groups ($n = 6$). Afterward, the mice in each group were *in situ* subcutaneously (s.c.) injected with of PBS (200 μ L), Tob ($0.89 \times$ MIC, 14.24 μ g/mL; 200 μ L), BF ($2.24 \times$ MIC, 17.89 μ g/mL; 200 μ L), physically mixed Tob + BF (with equal dose to TBN, containing 14.24 μ g/mL of Tob and 17.89 μ g/mL of BF; 200 μ L), and TBN (32.0 μ g/mL, containing 14.24 μ g/mL of Tob and 17.89 μ g/mL of BF; 200 μ L) at the skin infection sites once a day, respectively. The entire experiment was replicated three times.

Subsequently, the recovery process of the infected areas was photographed macroscopically. The infected areas, body weight, and survival rate of the mice were monitored throughout the treatment period. After 10 days of observation, the mice were sacrificed and the skin tissues at the infected sites were harvested for histological analysis of hematoxylin and eosin (H&E) and Masson's trichrome staining. To determine the number of bacteria at the infected sites, the infected tissues were homogenized in PBS and plated on TSA petri dishes (CFUs were measured by standard plate count method). The bacterial colonies were counted after 24 h incubation at 37 °C ($n = 3$).

In addition, the expression levels of representative inflammatory factors, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), in the skin and blood of mice were measured using high-sensitivity ELISA kits following the protocols recommended by the manufacturer, respectively.

In vivo therapeutic effect and infected skin recovery were further assessed by immunofluorescence staining. Co-localization of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and alpha smooth muscle actin (α -SMA) was performed to confirm regenerated capillaries and arterioles. The paraffin sections (5 μ m) of infected skin tissues were deparaffinized, washed 3 times in PBS (5 min), blocked with 5 % serum (30 min), and incubated with Anti-VEGFA antibody [VG-1] (mouse), anti-EGF antibody (rabbit), anti- α -SMA antibody (mouse), and anti-CD31 antibody (rabbit) at 4 °C, respectively. After being treated with TRITC goat anti-mouse IgG antibody and FITC goat anti-rabbit IgG antibody, the nuclei of the samples were stained with DAPI. The images were examined using a fluorescence microscope.

5.18. In vivo biomimetic responsive evaluation

To investigate the *in vivo* biomimetic responsive antibacterial ability of TBN, two type of animal models were created. Briefly, after removing the dorsal hair of Balb/c male mice, the mice were subcutaneously inoculated with MDRSA suspension (1×10^8 CFU/mL, 100 μ L). After 24 h injection, the mice with skin infection were randomly divided into two groups ($n = 3$). Then, MDRSA-infected mice were treated with *i.v.* injection of PBS and TBN (10 mg/kg) once a day, respectively, and the treatment lasted 12 days. The recovery process of the infected areas recorded the through macroscopic photographs.

Furthermore, we constructed the seconded animal model to deeply understand the biomimetic responsive antibacterial mechanisms of TBN. The healthy Balb/c mice were randomly divided into two groups ($n = 3$) and injected (*i.v.*) with PBS and TBN (10 mg/kg), respectively, followed by subcutaneous injection of MDRSA (1×10^8 CFU/mL, 100 μ L) at 24 h post inoculation. Afterward, we monitored the processes of the infected areas through macroscopic photographs.

5.19. In vivo biosafety analysis

Healthy Balb/c mice were randomly divided into 2 groups ($n = 3$) and injected (*i.v.*) with PBS and TBN (10 mg/kg) once a day, respectively. After 12 days of treatment, blood was drawn from the orbital

sinus for blood biochemistry testing, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CRE), total bilirubin (TBIL), and total protein (TP). Meanwhile, the internal organs (heart, liver, spleen, lung, kidney) of mice were collected and stained with H&E for histological examinations.

5.20. Statistical analysis

All data were presented as mean $\pm$ standard deviation. The images were processed by Image J software. The statistical significance of differences was determined by a two-tailed Student's *t*-test.

CRedit authorship contribution statement

Ya-nan Fu: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jiali Yang:** Validation, Investigation. **Lifei Huang:** Methodology, Investigation. **Fang Liu:** Investigation. **Fanqiang Bu:** Investigation. **Xiaoxu Kang:** Investigation. **Guofeng Li:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Qun Ren:** Writing – review & editing. **Thomas Fintan Moriarty:** Writing – review & editing, Conceptualization. **Xing Wang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

All animal procedures were approved for animal ethics and welfare permission from the Animal Ethics Committee of the China-Japan Friendship Hospital (the Approval no: zryhy12-20-08-3) and carried out under the supervision and evaluation of the SPF Animal Department of Clinical Institute in China-Japan Friendship Hospital. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to obtain statistically significant data.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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