Repurposing Synthetic Acetaminophen Derivatives Containing a Benzothiazole Scaffold as an Alternative Therapy for Infectious Diarrhea Caused by Drug-Resistant Shigella Species

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Abstract: Diarrhea remains one of the leading causes of mortality worldwide, especially among children. Accumulated evidence has shown that Shigella species are the most prevalent bacteria responsible for diarrhea in developing countries. Antimicrobial therapy is necessary for Shigella infections; however, the development of resistance against current drugs justifies the pressing need to search for alternative medications. In this study, we have applied antibacterial phenotypic screening to identify potent anti-Shigella compounds across a broad chemical diversity, including selected acetaminophen derivatives containing a benzothiazole backbone, and their combination with certain antibiotics. As a result, two acetaminophen derivatives containing a benzothiazole backbone (4a and 4b) inhibited the growth of Shigella flexneri with a common MIC value of 12.5 µg/mL. These compounds were established through a time-kill kinetics study to be potentially bactericidal. Meanwhile, the 2-aminobenzothiazoles (1a and 1b) used for the synthesis of compounds 4 (a and b) were found to be poorly active (MIC: 100 µg/mL) against this pathogen. Combination studies of 4a and 4b with the least effective antibiotics (ceftriaxone and cotrimoxazole) demonstrated synergistic anti-Shigella activity with MIC values decreasing from 12.5 to 0.781 µg/mL. The present study demonstrates that the azobenzothiazole dyes 4 (a and b) can be repurposed as potential anti-Shigella compounds, thus providing potential chemical pharmacophores for the discovery of drugs against infectious diarrhea caused by Shigella and other enteric pathogens, especially in developing countries.

Keywords: acetaminophen; benzothiazoles; azo dyes; Shigella flexneri; anti-Shigella activity; repositioning; combination studies; diarrhea

1. Introduction

Diarrhea is among the manifestations of gastrointestinal infections caused by a number of bacterial, viral and parasitic microorganisms [1,2]. The release of at least three watery or loose stools per day, or more often (discharge of 10 and 200 g/kg/day of feces in infants and adults, respectively) than usual is generally termed as diarrhea [2,3]. Contaminated food or drinking water and poor sanitation are associated with the transmission of infectious diarrhea [4,5]. In developing countries, this disease is ranked among the top three leading causes of death in children aged below five years [2,5]. Recent estimates point out approximately 1.7 billion cases of childhood diarrhea annually with 525,000 deaths in children under five years old, with a high concentration in sub-Saharan Africa and South Asia [2,6]. Notably, gram-negative bacteria of the genus Shigella account for 69% of all episodes and 61% of all deaths caused by bacterial diarrhea [7,8]. One such bacterial diarrhea includes...
shigellosis, which is caused by *Shigella* species, such as *Shigella flexneri*, *Shigella sonnei* and *Shigella boydii* [9]. Most of the breakouts of shigellosis have been ascribed to *S. flexneri* [10]. Clinical cases of diarrhea caused by *Shigella flexneri* have also been reported among patients with primary HIV infection [11,12]. *Shigella* species are primarily spread through the fecal–oral route, whereas the housefly *Musca domestica* acts as a vector for their transmission [13]. As low as 10–100 bacterial counts can potentially cause diarrheal infection [14,15]. The prevention and treatment of this infection is economically influential, especially in the developing world [16,17]. Current treatments for diarrhea include the use of antimicrobials, especially for diarrhea caused by bacteria [5]. However, most of these therapies have lost their efficacy due to the spread of multi-drug-resistant *Shigella*, mostly in infants and patients with HIV [18]. Consequently, there has been an extensive use of antimicrobials, such as ciprofloxacin, azithromycin, pivmecillinam, ampicillin and ceftriaxone (as per the WHO recommendations) as the empirical drug choices for the treatment of infection by fluoroquinolone-resistant *Shigella* species [19,20]. In fact, treatment options for shigellosis include the use of the following families of antimicrobials: β-lactams, cephalosporins and fluoroquinolones [20]. A decline in membrane permeability, removal of drugs by efflux pumps and target mutational changes are among the mechanisms of resistance to these medications by *Shigella* species [21]. Therefore, the search for effective and safe antidiarrheal drugs is valuable. Notably, the rapid development of bacterial resistance has prompted numerous researchers to investigate drugs with healing outcomes other than antimicrobial action. Such investigations include the antibacterial activity of phenothiazines, non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetics, statins, antiplatelets and antidepressants [22]. It has also been demonstrated that acetaminophen displays anti-biofilm activity, thus prompting the interest in repurposing this drug for the treatment of bacterial infections [23,24]. Lagadinou et al. [22] have also reported the inhibitory effects of acetylsalicylic and salicylic acids against *Helicobacter pylori*, *Campylobacter pylori* and *Klebsiella pneumoniae* and *Epidermophyton floccosum*, *Microsporum* and certain *Trichophyton* species. Acetaminophen is one of the most widely used analgesic, anti-inflammatory and antipyretic drugs [25] that has been reported to exhibit antimicrobial activity vis-à-vis bacteria, such as *Staphylococcus aureus* [26]. Although the use of this medicine has been linked to liver failure, hepatotoxicity occurring with acetaminophen is typically correlated with high doses that exceed the recommended maximum dose [27,28]. However, selected structural modifications of acetaminophen with the benzothiazole ring have afforded pharmacologically active compounds with decreased nephrotoxicity as compared to the parent molecule, i.e., acetaminophen [29]. On the other hand, the antibacterial potential of benzothiazoles is undeniable, as an important number of recent reports have described their efficacy against pathogenic microbes via numerous mechanisms of action, such as the inhibition of reactive chemical species [30], inhibition of uridine diphosphate-n-acetyl enol pyruvyl glucosamine reductase [31], DNA gyrase inhibition [32,33], peptide deformylase inhibition [34], aldose reductase inhibition [35], dihydroorotase inhibition [36], casdihydrofolate reductase inhibition [37], enoyl acyl carrier protein reductase inhibition [38], dialkyglycine decarboxylase inhibition [39], dehydrosqualene synthase inhibition [40], DNA gyrase and tyrosine kinase inhibition [41] and dihydropteroate synthase inhibition [42] among others [43].

Interestingly, the nephrotoxicity caused by an overdose of acetaminophen is often averted when this drug harbors the benzothiazole ring [44]. In addition, benzothiazole-based compounds have also been reported to exhibit antibacterial activity [45–48]. Furthermore, antibiotic combination therapy has been argued as a forefront strategy to overcome bacterial drug resistance to antibiotics [49]. Although few studies have revealed the effectiveness of acetaminophen against *Staphylococcus aureus* [26], no report has shown the mechanistic basis of the antibacterial action of acetaminophen and its derivatives against *Shigella* species, the pathogens responsible for infectious diarrhea.

In continuation of our search for new scaffolds and better combinations with antimicrobial efficacy from existing therapies, this study aimed to repurpose acetaminophen from its traditional use as an anti-inflammatory drug to a potentially active hit compound
against *Shigella*-causing diarrhea. Herein, a series of diazobenzothiazoles dyes and their combination with selected antibiotics were screened for anti-shigellosis activity against a panel of *Shigella* spp., *viz.* *Shigella sonnei* NR 519, *Shigella boydii* NR 521, *Shigella flexneri* NR 518 and *Shigella dysenteriae*. A plausible antibacterial mechanism of action of the most active compounds was also elucidated.

2. Materials and Methods

2.1. Chemistry

2.1.1. General Information

Melting points were measured on a Buchi melting point apparatus. Ultraviolet (UV)-visible absorption spectra were registered in methanol using sample solutions of concentration 5 × 10⁻⁵ mol/L with a Beckman DU-640 spectrophotometer (Beckman-Coulter, Brea, CA, USA). FT-IR spectra were measured with a Fourier transform infrared spectrometer JASCO FT/IR-4100 and a Perkin Elmer FT-IR 2000 spectrometer. NMR spectra were recorded at 25 °C on a 400 MHz spectrometer NMR Bruker Advance 400 at 400 MHz for ¹H and 100 MHz for ¹³C, using DMSO-d₆ as a solvent and TMS as the internal standard. Elemental analyses were performed with a Euro EA CHNSO analyzer from Hekatech Company, Wegberg, Germany. Thin-layer chromatography was performed on Eastman Chromatogram Silica Gel Sheets (13.181; 6.060) with fluorescent indicators using hexane and ethyl acetate (4:6) as the mobile phases.

2.1.2. Preparation of the Reagents and Starting Materials

All reagents were obtained from commercial sources and used without further purification.

a. Preparation of diazonium salt solution

A diazonium solution was prepared as previously described [44]. Firstly, compound 1 (10 mmol) was dissolved in DMSO (10 mL) and cooled to 0–5 °C. Secondly, a mixture of 10 mmol) was dissolved in DMSO (10 mL) and cooled to 0–5 °C. Then, 15 mL of sodium acetate solution (10%) was added to the preparation to obtain a final pH for the solution between 9 and 11. The precipitate formed was filtered and recrystallized from methanol.

2.1.3. Preparation of the Reagents and Starting Materials

b. General procedure for the synthesis of 4a and 4b

The as-prepared diazonium solution was slowly added at 0–5 °C to a solution of acetaminophen 3, which was obtained by dissolving 1.51 g (10 mmol) of drug in DMSO (10 mL). The mixture was kept cooled in the ice bath and stirred occasionally for 1 h and then 15 mL of sodium acetate solution (10%) was added to the preparation to obtain a final pH for the solution between 9 and 11. The precipitate formed was filtered and recrystallized from methanol.

N-(2-(6-ethoxy-5-(5-((6-ethoxybenzo[d]thiazol-2-yl)diazonyl)benzo[d]thiazol-2-yl)diazonyl)-3-(6-ethoxybenzo[d]thiazol-2-yl)diazonyl)-5,6-bis((6-ethoxybenzo[d]thiazol-2-yl)diazonyl)-4-hydroxyphenyl)acetamide hexahydrate (4a) was obtained in 47% yield as red powder; m.p. 197–198 °C (dec); (lit. 197–199 °C (dec); Tseneugne et al. [44]; ¹H-NMR (DMSO-d₆, 400 MHz): δ 8.11 (d, 1H, = J = 2.8, H-4⁺), 8.00 (s, 1H, H-7), 8.02 (s, 1H, H-7⁺), 7.91 (d, 1H, = J = 9.2, H-4⁻), 7.71 (d, 1H, = J = 3.6, H-7⁻), 7.65 (d, 2H, = J = 8.8, H-4⁻ and H-4⁺), 7.62 (d, 1H, = J = 6.4, H-7⁻), 7.61 (s, 1H, H-4⁻), 7.58 (s, 1H, H-4⁺), 7.16 (dd, 2H, = J = 9.2 and 2.8, H-5⁻ and H-5⁺), 7.15 (d, 1H, = J = 2.4, H-7⁻), 7.09 (d, 2H, = J = 8.8, H-5⁻ and H-5⁺), 4.18, 4.16, 4.14, 4.12, 4.11, 4.10 (12H, OCH₂CH₃), 2.05 (s, 3H, COCH₃), 1.58, 1.40, 1.38, 1.36, 1.35 (18H, OCH₂CH₃); ¹³C-NMR (DMSO-d₆, 100 MHz): δ 199.1 (CO); 178.3 (C-2 and C-2⁺); 174.5 (C-2⁻); 173.7 (C-2⁺); 173.4 (C-2⁻); 173.2 (C-2⁺); 163.6 (C-5⁻); 163.3 (C-5⁺); 161.8 (C-5⁻); 161.7 (C-5⁺); 161.4 (C-5⁻); 158.5 (C-5⁺); 157.2 (C-3a⁻); 156.9 (C-3a⁺); 152.0 (C-3a and C-3a⁺); 151.0 (C-3a⁺); 143.6 (C-2⁻); 141.8 (C-3⁺); 141.5 (C-4⁻); 141.1 (C-6⁻); 140.9 (C-6⁺); 137.4 (C-4a); 137.3 (C-4a⁺); 136.9 (C-5⁻); 136.5 (C-1⁻); 130.4 (C-4a⁻ and C-6⁻); 128.7 (C-4a⁺); 123.9 (C-3⁻); 123.8 (C-7⁺); 123.5 (C-6⁺); 122.1 (C-6⁻); 121.7 (C-6⁺); 121.4 (C-6⁻); 120.9 (C-7⁺); 120.7 (C-7⁻); 117.2
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2.2. Biological Activity

2.2.1. Reference Compounds and Bacterial Strains

The reference compounds [ciprofloxacin (antibiotic) and triton X (standard hemolytic agent)] were used as positive controls in this study were acquired from Sigma Aldrich (St. Louis, MO, USA). Other antibiotics that were used in combination studies included ampicillin (ampicillin sodium for IM/IV injection, Shanxi Xinyitong Pharmaceutical Co. Ltd., Jinzhong, China), cefixime (CEFLIXE tablets, Farma hub, Haridwar, India), co-trimoxazole (oral tablets, Africure Pharmaceutical Ltd., Bwang Bakoko, Yassa-Douala, Cameroon) and tetracycline (tetracycline hydrochloride tablets, Zhejiang Cheng Yi Pharmaceutical Co. Ltd., Wenzhou, China) and were obtained from local drugstores in Yaoundé, Cameroon. For the antibacterial tests, four bacterial strains were used in this study: Shigella flexneri (donated by the Centre Pasteur of Cameroon). These bacterial species were sub-cultured at 35 ± 2 °C for 24 h on Mueller–Hinton agar prior to each experiment.

2.2.2. Antibacterial Activity

a. Preparation of stock solutions

The stock solutions were obtained by dissolving 10 mg of each test compound or antibiotic (other than ciprofloxacin) in 1 mL of dimethyl sulfoxide (DMSO) to yield a final concentration of 10 mg/mL. Ciprofloxacin (positive control) solution was prepared at 1 mg/mL by dissolving 1 mg of drug in 1 mL of acidified water (HCl 0.5 N). These solutions were stored at 4 °C until further use.

b. Preparation of bacterial inocula
The bacterial suspensions were prepared at $1.5 \times 10^8$ CFU/mL with reference to the 0.5 McF standard by introducing a colony from 18 to 24 h old cultures on Mueller–Hinton agar into 10 mL of NaCl 0.9% and further calibrated by turbidity comparison.

c. Determination of minimum inhibitory concentrations

The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) referring to the protocol number M09-A7 of the Clinical & Laboratory Standards Institute (CLSI) guidelines [50] with minor changes. In brief, 196 µL of Mueller–Hinton broth (MHB) was distributed to the first wells, whereas 100 µL of the same solution was introduced into the rest of wells. Next, 4 µL of compound solutions (10 mg/mL) was added to the corresponding wells followed by a two-fold serial dilution. Afterward, 100 µL of bacterial suspension ($10^6$ CFU/mL) was introduced into wells, with the exception of the sterility control wells. The final concentrations ranged from 100 to 3.125 µg/mL and from 0.25 to 0.0078 µg/mL, for compounds and ciprofloxacin, respectively, with 200 µL as the final volume. Next, the plates were incubated at 37 °C for 24 h. After the incubation period had elapsed, 20 µL of resazurin’s solution (0.15 mg/mL) was added to each well with a subsequent incubation at 37 °C for 30 min. The test was carried out in triplicate in sterile 96 well microplates. The MICs were determined as the lowermost concentrations of test compounds that did not induce color change from blue to pink (consistent to no visible growth of bacteria).

2.2.3. Cytotoxicity Assay

The cytotoxic effect of compounds and compound–antibiotic combinations was evaluated on the erythrocyte membrane by using a spectrophotometric method based on hemoglobin release [51]. Hemolytic effect was evaluated by the treatment of normal red blood cells with test compounds in RPMI 1640 culture medium without phenol red. Firstly, the erythrocyte suspension was prepared according to a previously reported protocol by Fidock et al. [52] with a few changes. Blood from an O+ donor was centrifuged at 3500 rpm for 5 min, and after the removal of the plasma, the pellet was washed 3 times under the same conditions. The remaining pellet was suspended with RPMI 1640 culture medium to obtain a hematocrit of 4%. Five hundred microliters of this suspended pellet were added to microtubes having test compounds at different concentrations (200–12.5 µg/mL). RPMI 1640 medium (for baseline values) and 0.5% Triton X-100 in RPMI 1640 (for 100% hemolysis) were used as controls. After incubation at 37 °C for 3 h in a 5% CO₂ atmosphere, the preparations were centrifuged at 2500 rpm for 5 min and 200 µL of the supernatant was introduced into a 96-well sterile culture plate for the determination of the hemolytic effect. To evaluate the hemoglobin release, the absorbance of each well was measured at 540 nm using a microplate reader (TECAN Infinite M200, Männedorf, Switzerland). The test was performed in triplicate. From the obtained optical densities, the percentage of hemolysis was calculated using the following formula:

\[
\% \text{ Hemolysis} = \frac{(\text{Absorbance of test compounds} - \text{Absorbance of blank})}{(\text{Absorbance of positive control})} \times 100
\]

Using Graphpad Prism 8.0.1 software (San Diego, CA, USA), median hemolytic concentrations ($HC_{50}$) were determined from concentration–response sigmoidal curves obtained by plotting the percentage of hemolysis against the decimal logarithm of concentration. Selectivity indices were further determined for each test substance as follows:

\[
\text{Selectivity Index (SI)} = \frac{HC_{50}}{MIC_{50}} \text{ (Shigella flexneri)}
\]

With $MIC_{50} = MIC/2$ [53].
2.2.4. Potential Mechanism of Antibacterial Action and Combination Studies

a. Bacterial time–kill kinetics

The kinetics of bacterial mortality were studied on the most active antibacterial compounds (4a and 4b) according to Klepser et al.’s protocol [54] with minor modifications, with the use of opacimetry based on the turbidity of cell suspensions as a function of bacterial load rather than colony counts on agar. The assays were performed in triplicate in 96-well plates at sub-inhibitory, inhibitory and supra-inhibitory concentrations. Indeed, a serial dilution was performed for each sample to obtain 4 MIC, 2 MIC, MIC, 0.5 MIC and 0.25 MIC. Afterward, 100 µL of Shigella suspension (10^6 CFU/mL) was introduced in the wells of the plate, with the exception of the sterility control wells. The negative control’s well was made up of culture media and bacterial suspension, whereas the positive control’s well comprised culture media, bacterial suspension and ciprofloxacin. Next, the preparations were incubated at 37 °C for 24 h. Within this time period, the bacterial growth kinetics were studied by measuring optical densities at 620 nm and at 0, 1, 2, 4, 6, 8, 10, 12 and 24 h using a TECAN microtiter plate reader (Infinite M200, Männedorf, Switzerland) against the blank (compound in culture media). The results allowed us to plot the optical density curves as a function of the incubation times and these curves were used to determine the minimum time at which the inhibitory effect was first observed and the bactericidal and bacteriostatic effects of the compounds, as well as the time of the re-emergence of the bacterial species. The minimum bactericidal concentrations (MBCs) were determined as the concentrations that induced a continuous decrease in the bacterial population.

b. Combination of active compounds with selected antibiotics and antibacterial studies

b1. Preparation of the intermediate plate

Intermediate plates were obtained from the as-prepared stock solutions of the most active compounds and the least effective antibiotics (10 mg/mL) by a serial dilution of test samples with MHB in two different microplates. The concentrations ranged from 4 MIC to MIC/32 in the wells (from row A to H) and for compound and antibiotic (from 1 to 8), respectively, and the volumes were topped up to 100 µL.

b2. Determination of fractional minimum inhibitory concentration (FICI) indices

The combined effect of compounds with least effective antibiotics was assessed using a checkerboard dilution method [55]. Twenty-five microlitres (25 µL) of various concentrations of test compounds, which were withdrawn from the intermediate plate, were added to a 96-well microplate containing MHB (50 µL) in order to obtain compound and antibiotic at different concentrations in the considered wells (MIC/1 in well 1 to MIC/128 in well 8 and MIC/1 in well A to MIC/128 in well H, respectively). Next, 100 µL of bacteria suspension (10^6 CFU/mL) was added to the test wells and negative control for a final load of 5 × 10^5 CFU/mL. After the incubation of the plates at 37 °C for 24 h, 20 µL of resazurin (0.15 mg/mL) was added to each well, followed by further incubation for 30 min. Ciprofloxacin was used as a positive control. For each sample, the experiment was carried out in triplicate.

The fractional minimum inhibitory concentrations indices (FICI) of different combinations corresponding to the wells with no visible bacterial growth were calculated using the following formula:

\[ FICI = FICA + FICB \]
\[ FICA = \frac{MIC_{A_{\text{com}}}}{MIC_A} \quad \text{and} \quad FICB = \frac{MIC_{B_{\text{com}}}}{MIC_B} \]

\[ A = \text{compound}; \ B = \text{antibiotic} \quad \text{and} \quad \text{com} = \text{in combination.} \]

The type of the compound’s interaction was interpreted using FICI values. For FICI values ≤ 0.5, the interaction is synergistic, whereas for 0.5 < FICI ≤ 1.0, the interaction is additive. For 1.0 < FICI ≤ 4.0, there is no interaction, while FICI values > 4.0 indicate antagonistic interactions. In addition, the type of interaction involved was better appreciated geometrically using isobolograms, obtained by plotting the FIC values of a compound (FICA) in combination against those of the antibiotic (FICB) (Figure 1).
2.2.5. Data Analysis

In this study, results were presented as the mean ± standard deviation. Quantitative variables were subjected to one-way analysis of variance (ANOVA) followed by multiple comparisons for significant differences using the Dunnett test at $p < 0.05$. As already discussed in Section 2, MIC and HC$_{50}$ values were derived from graphs plotted using Graph Pad prism version 8.1.0 (Washington, DC, USA). The graphical representation of data was carried out using Microsoft Excel 2016.

3. Results

3.1. Chemistry

The diazonium ions 2 were readily synthesized according to the literature [44] by adding 2-aminobenzothiazole 1 to nitrosyl sulfuric acid at 0–5 °C (Scheme 1).

\[
\begin{align*}
& \text{1a: } R^1 = H, \ R^2 = \text{OCH}_2\text{CH}_3 \\
& \text{1b: } R^1 = H, \ R^2 = \text{OCH}_3 \\
\end{align*}
\]

Scheme 1. Chemical reactions leading to the formation of the diazonium intermediates 2.

The azobenzothiazole dyes 4 (a and b), which were used in this study, were obtained as a result of the coupling reactions of p-acetaminophen 3 with the diazotized 2-aminobenzo[d]thiazole derivatives 2, referring to previously reported procedures [44] as depicted in Scheme 2.

Data obtained for yields and melting points, as well as for all of the spectroscopic data for compounds 4a and 4b, agree with those originally reported [44].
Scheme 2. Reaction sequences to obtain compounds 4a and 4b.

3.2. Antibacterial and Cytotoxic Assays

The results obtained from the antimicrobial activity of 2-aminobenzothiazole derivatives 1 (a and b), acetaminophen 3 and diazobenzothiazoles dyes 4 (a and b), are summarized in Table 1. The minimum concentrations that inhibited the bacterial growth ranged from 12.5 to 100 µg/mL for test compounds and from <0.062 to 12.5 µg/mL for reference antibiotics. Compounds 4a and 4b were recorded as the most active compounds (MIC = 12.5 µg/mL on S. flexneri and 50 µg/mL on S. sonnei), whereas compound 3 was the least active compound (MIC > 100 µg/mL). Shigella flexneri was the most susceptible bacterial species, whereas S. boydii and S. dysenteriae were found to be the most resistant ones as no activity was observed with any of the test compounds (MIC > 100 µg/mL).

The susceptibility of the most sensitive strain viz. Shigella flexneri was evaluated against four antibiotics. Ampicillin and tetracycline were the most active antibiotics (MIC < 0.062 µg/mL), followed by cotrimoxazole and ceftriaxone with a common MIC...
value of 12.5 µg/mL. In the hemolysis test, compounds 4a and 4b afforded HC$_{50}$ values of 148.85 and 87.52 µg/mL, to yield selectivity indices (SI) of 23.68 and 13.92, respectively, inferring the non-toxicity of these compounds toward red blood cells. The other compounds (1a, 1b and 3) showed minimal hemolysis percentages (below 1% at 200 µg/mL), when compared with a standard hemolytic agent (triton X-100, 100% hemolysis). However, the selectivity indices were greater than 13.92 for all of the tested compounds (1a, 1b, 3, 4a and 4b), inferring that these compounds are non-toxic to red blood cells. These results demonstrated that 4a and 4b, which were found to be the most active compounds, were selective as they did not display toxicity on the membrane’s red blood cells. Thus, compounds 4a and 4b were further selected for bacterial growth kinetic assays.

### Table 1. Antimicrobial activity (MIC in µg/mL), cytotoxic effect (HC$_{50}$ in µg/mL) on red blood cells and selectivity indices of test compounds and selected antibiotics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Minimum Inhibitory Concentrations (µg/mL)</th>
<th>HC$_{50}$ (µg/mL)</th>
<th>SI (HC$<em>{50}$/MIC$</em>{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF NR 518</td>
<td>SO NR 519</td>
<td>SB NR 521</td>
</tr>
<tr>
<td>Compounds</td>
<td>1a</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>100</td>
<td>-</td>
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<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4a</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Amp</td>
<td>&lt;0.062</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Cef</td>
<td>12.5</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Tet</td>
<td>&lt;0.062</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Cot</td>
<td>12.5</td>
<td>/</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.062</td>
<td>0.015</td>
<td>0.062</td>
</tr>
</tbody>
</table>

Amp: ampicillin; Cef: ceftriaxone; Cot: cotrimoxazole; HC$_{50}$: median hemolytic concentration; /: not determined; SB: Shigella boydii; SD: Shigella dysenteriae; SF: Shigella flexneri; SI: selectivity index; <: >100 µg/mL; SO: Shigella sonnei; Tet: tetracycline.

3.3. Bacterial Growth Kinetics and Combination Studies

3.3.1. Shigella Growth Kinetics

Figure 2 shows the variation of the bacterial population upon treatment with the compounds as a function of time. It appears that the bacterial inhibition is dose-dependent and is observed after a minimum incubation time of 2 h. In addition, a continuous decrease in the optical densities corresponding to a reduction in the bacterial load was observed with 2MIC and 4MIC as compared to ciprofloxacin, thus suggesting a bactericidal effect (MBC = 25 µg/mL) of both the compounds 4a and 4b.

3.3.2. Antibacterial and Cytotoxic Activities of the Combination of Active Compounds with Selected Antibiotics

a. Fractional minimum inhibitory concentration indices (FICIs)

The most active antibacterial compounds (4a and 4b), and the least active antibiotics (cotrimoxazole and ceftriaxone), were selected for combination studies. Table 2 shows the synergistic interaction for all combinations, according to the calculated values of fractional minimum inhibitory concentrations (FICI ≤ 0.5), meaning a mutual potentiation of both azo-compounds and antibiotics (reduction in MIC value from 12.5 µg/mL to 0.097 µg/mL). The isobologram plotted from 4a FIC values versus ceftriaxone in Figure 3 has a convex appearance with a large number of points below the coordinate line (0.5-0.5), confirming the observed antibacterial effect.
Figure 2. Bacterial time–kill curves of *S. flexneri* NR518 following incubation with compounds 4a and 4b. Data are presented as the mean ± standard deviation. The curves of the figure that are assigned to the stars are significantly different, (*) relates to $p < 0.05$, (**) correlates with $p < 0.01$ and (***) corresponds to $p < 0.001$ (Dunnett test). 4a: N-(2-(6-Ethoxy-5-((6-ethoxybenzo[d]thiazol-2-yl)diazenyl)benzo[d]thiazol-2-yl)diazenyl)-3-(6-ethoxy-5-((6-ethoxybenzo[d]thiazol-2-yl)diazenyl)benzo[d]thiazol-2-yl)diazenyl)-5,6-bis(6-ethoxybenzo[d]thiazol-2-yl)diazenyl)-4-hydroxyphenyl) acetamide hexahydrate; 4b: N-(4-Hydroxy-3-((6-methoxybenzo[d]thiazol-2-yl)diazenyl)phenyl)acetamide hexahydrate; MIC: minimum inhibitory concentration; NC: negative control; PC: positive control (ciprofloxacin, 0.25 µg/mL).
Table 2. Fractional minimum inhibitory concentration indices (FICIs) of various combinations of compounds with antibiotics against *Shigella flexneri* NR518.

(a) FICI of Different Combinations of 4a and Ceftriaxone against *Shigella flexneri* NR518

<table>
<thead>
<tr>
<th>Corresponding MIC Wells</th>
<th>Conc. 4a (µg/mL)</th>
<th>Conc. Cef (µg/mL)</th>
<th>FIC 4a</th>
<th>FIC Cef</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>3.125</td>
<td>0.997</td>
<td>0.25</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>G4</td>
<td>1.562</td>
<td>0.195</td>
<td>0.13</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>F5</td>
<td>0.781</td>
<td>0.390</td>
<td>0.06</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>E5</td>
<td>0.781</td>
<td>0.781</td>
<td>0.06</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>F6</td>
<td>0.390</td>
<td>0.390</td>
<td>0.03</td>
<td>0.03</td>
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</tr>
<tr>
<td>D7</td>
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<td>1.562</td>
<td>0.02</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>D8</td>
<td>0.097</td>
<td>1.562</td>
<td>0.01</td>
<td>0.13</td>
<td>0.13</td>
</tr>
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</table>

Mean FICI (Interaction) 0.14 (S)

(b) FICI of different combinations of 4a and cotrimoxazole against *Shigella flexneri* NR518

<table>
<thead>
<tr>
<th>Corresponding MIC Wells</th>
<th>Conc. 4a (µg/mL)</th>
<th>Conc. Cot (µg/mL)</th>
<th>FIC 4a</th>
<th>FIC Cot</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
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<td>0.097</td>
<td>0.25</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>G3</td>
<td>3.125</td>
<td>0.195</td>
<td>0.25</td>
<td>0.02</td>
<td>0.27</td>
</tr>
<tr>
<td>F4</td>
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<td>0.13</td>
<td>0.03</td>
<td>0.16</td>
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<tr>
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<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>E6</td>
<td>0.390</td>
<td>0.781</td>
<td>0.05</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>E7</td>
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<td>0.781</td>
<td>0.02</td>
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<td>0.08</td>
</tr>
<tr>
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<td>1.562</td>
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<td>0.13</td>
<td>0.13</td>
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</tbody>
</table>

Mean FICI (Interaction) 0.16 (S)

(c) FICI of different combinations of 4b and cotrimoxazole against *Shigella flexneri* NR518

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<thead>
<tr>
<th>Corresponding MIC Wells</th>
<th>Conc. 4b (µg/mL)</th>
<th>Conc. Cot (µg/mL)</th>
<th>FIC 4b</th>
<th>FIC Cot</th>
<th>FICI</th>
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</thead>
<tbody>
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<td>0.195</td>
<td>0.250</td>
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<td>0.016</td>
<td>0.141</td>
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<tr>
<td>G5</td>
<td>0.781</td>
<td>0.391</td>
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<td>0.016</td>
<td>0.078</td>
</tr>
<tr>
<td>F6</td>
<td>0.391</td>
<td>0.391</td>
<td>0.031</td>
<td>0.031</td>
<td>0.063</td>
</tr>
<tr>
<td>E7</td>
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<td>0.016</td>
<td>0.031</td>
<td>0.047</td>
</tr>
<tr>
<td>E8</td>
<td>0.098</td>
<td>0.391</td>
<td>0.008</td>
<td>0.031</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Mean FICI (Interaction) 0.127 (S)

(d) FICI of different combinations of 4b and Ceftriaxone against *Shigella flexneri* NR518

<table>
<thead>
<tr>
<th>Corresponding MIC Wells</th>
<th>Conc. 4b (µg/mL)</th>
<th>Conc. Cef (µg/mL)</th>
<th>FIC 4b</th>
<th>FIC Cef</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>3.125</td>
<td>0.097</td>
<td>0.25</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>G3</td>
<td>3.125</td>
<td>0.195</td>
<td>0.25</td>
<td>0.02</td>
<td>0.27</td>
</tr>
<tr>
<td>F4</td>
<td>1.562</td>
<td>0.390</td>
<td>0.13</td>
<td>0.03</td>
<td>0.16</td>
</tr>
<tr>
<td>E5</td>
<td>0.781</td>
<td>0.781</td>
<td>0.06</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>E6</td>
<td>0.390</td>
<td>0.781</td>
<td>0.03</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>E7</td>
<td>0.195</td>
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<td>0.02</td>
<td>0.06</td>
<td>0.08</td>
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<tr>
<td>E8</td>
<td>0.097</td>
<td>0.781</td>
<td>0.01</td>
<td>0.06</td>
<td>0.07</td>
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</tbody>
</table>

Mean FICI (Interaction) 0.15 (S)

Capital letters with a number indicate the wells representing the different pairs of concentrations considered as MICs; 4a: N-(2-(6-Ethoxy-5-((6-ethoxybenzo[d][1,2,4]thiazol-2-yl)diazenyl)benzo[d][1,2,4]thiazol-2-yl)diazenyl)-3-(6-ethoxy-5-((6-ethoxybenzo[d][1,2,4]thiazol-2-yl)diazenyl)benzo[d][1,2,4]thiazol-2-yl)diazenyl)-4-hydroxyphenyl) acetamide hexahydrate; 4b: N-(4-Hydroxy-3-((6-methoxybenzo[d][1,2,4]thiazol-2-yl)diazenyl)phenyl)acetamide hexahydrate; Cef: ceftriaxone; Conc: concentration (µg/mL); Cot: cotrimoxazole; FIC: fractional minimum inhibitory concentration; FICI: fractional minimum inhibitory concentration index; S: synergistic effect.
b. Minimum inhibitory concentrations and selectivity indices of drug combinations

For each antibiotic–compound tandem chosen for MIC confirmation, the combinations which displayed the lowest FICI values were as follows: 4a-cot (0.195 and 0.781 µg/mL, respectively), 4a-cef (0.390 and 0.390 µg/mL, respectively), 4b-cot (0.098 and 0.391 µg/mL, respectively) and 4b-cef (0.097 and 0.781 µg/mL, respectively). Table 3 summarizes the MIC values that ranged from 0.625 (4b-cef) to 2.5 µg/mL (4a-cot), confirming compound 4b as the best potential candidate for combination with both ceftriaxone and cotrimoxazole. Upon hemolytic studies, all combinations showed hemolytic percentages below 1% at 200 µg/mL, which value is negligible compared to the percentage obtained with the standard hemolytic agent triton X-100 (100% hemolysis). Moreover, the combinations of 4a and 4b with the antibiotics (ceftriaxone and cotrimoxazole) selectively (SI: >80) eliminated the bacteria without causing any harm to red blood cells.

Table 3. Minimum inhibitory concentrations of the most potent drug combinations on S. flexneri NR 518.

<table>
<thead>
<tr>
<th>Combinations</th>
<th>MIC (µg/mL) on S. flexneri</th>
<th>HC50 (µg/mL)</th>
<th>SI (HC50/MIC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a-Cot</td>
<td>2.5</td>
<td>&gt;200</td>
<td>&gt;80</td>
</tr>
<tr>
<td>4a-Cef</td>
<td>1.25</td>
<td>&gt;200</td>
<td>&gt;160</td>
</tr>
<tr>
<td>4b-Cot</td>
<td>0.625</td>
<td>&gt;200</td>
<td>&gt;320</td>
</tr>
<tr>
<td>4b-Cef</td>
<td>0.625</td>
<td>&gt;200</td>
<td>&gt;320</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.062</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>


4. Discussion

In this study, we report the antibacterial activity of acetaminophen hybrid compounds against a variety of Shigella species that cause diarrhea in humans. As a result, the diazobenzothiazole dyes 4a and 4b exhibited a common MIC value of 12.5 µg/mL, which qualifies these compounds as active anti-Shigella compounds with reference to reported threshold values for active antimicrobial potential compounds [56]. By contrast, the 2-aminobenzothiazoles 1a and 1b were poorly active against S. flexneri with a common MIC value of 100 µg/mL, whereas acetaminophen (MIC value: >100 µg/mL) alone did not...
inhibit the growth of the *Shigella* species. The diazobenzothiazole backbone potentiated the antibacterial activity of acetaminophen. As already discussed, several benzothiazole-based compounds were found to inhibit the growth of a number of Gram-negative bacteria [44]. In reported studies, plausible antibacterial mechanistic studies of benzothiazole-based compounds include (i) interference with cell wall synthesis, (ii) interference with nucleic acid synthesis, (iii) the inhibition of DNA replication or protein synthesis, (iv) the inhibition of biosynthetic pathways of essential compounds in bacterial cells and (v) the disruption of membrane function and integrity, among others [36,57]. Furthermore, the incubation of *Shigella* with the active compounds showed concentration- and time-dependent effects [58]. At 4 MIC, compounds 4a and 4b suggested a bactericidal orientation as there was a continuous decrease in the bacterial population from 0 to 24 h. Similarly, the treatment of *Shigella* cells with the positive control ciprofloxacin showed a bactericidal inclination. On the other hand, treatment at MIC showed almost similar trends like in 2 MIC, although complete bacterial elimination was not achieved after 24 h of incubation as evidenced by the curve for MICs that did not overlap with the X axis. By contrast, MIC/2 and MIC/4 did not inhibit the bacterial growth as evidenced by the increased trend of the curves at these concentrations. These results are consistent with the antibiotic power (Pa = MBC/MIC: 25/12.5 = 2) obtained for both compounds 4a and 4b, which classify them as bactericidal according to previously reported studies [59,60] that elaborate bactericidal (1 ≤ MBC/MIC ≤ 2) and bacteriostatic (4 ≤ MBC/MIC ≤ 16) effects of potential antimicrobial compounds based on the ratio MBC/MIC. Although the turbidity method used in this study appears to be an easier and faster method than the viable plate count approach, it has low accuracy since the optical densities poorly correlate with the expected bacterial counts [61]. The bactericidal trend of antibiotics vis-à-vis Gram- and Gram+ bacteria was previously attributed to the overproduction of the hydroxyl free radicals [62]. The existence of drug-resistant *Shigella* species has compelled many scientists to research combination therapies that use two or more antibiotics with the goal of obtaining an enhanced antibacterial effect (synergistic activity) [63,64]. Indeed, as an expert opinion stated when conducting a review published in the Expert Review of Anti-infective Therapy, Coates et al. [63] found antibiotic combination-based therapy, exploiting synergies and old-drug rejuvenation, for the decrease in resistance, as promising solutions to ampicillin resistance. From susceptibility assays, *S. flexneri* appeared as moderately resistant (MIC ≥ 8 µg/mL) towards cotrimoxazole and ceftriaxone (common MIC = 12.5 µg/mL), referring to the classification criteria published by Walsh [65]. Nguena-Dongue et al. [59] arrived at the same conclusion when evaluating the potentiation effect of mallotojaponin B on chloramphenicol against methicillin-resistant *Staphylococcus aureus*. Several authors reported the resistance of *S. flexneri* to both cotrimoxazole and ceftriaxone [7,13]. A combinatory assessment of compounds 4a and 4b with cotrimoxazole and ceftriaxone using a checkerboard method revealed a synergistic effect (mean FICI = 0.14) with significant MIC reduction (from 12.5 µg/mL to 0.097 µg/mL) suggesting a mutual potentiation of both compounds and antibiotics [35]. In a previous report by Nguena-Dongue et al., a combination of mallotojaponin B with chloramphenicol could significantly increase its antibacterial effect, thus reducing the MIC value from 12.5 to 0.781 µg/mL [59]. The optimal ratio combinations (with the lowest FICI) exhibited MIC values ranging from 0.625 (4b-cef) to 2.5 µg/mL (4a-cot) against *S. flexneri*, confirming the 4b derivative as the best candidate for combination with both the ceftriaxone and cotrimoxazole. Several authors have reported the synergistic effect of cotrimoxazole and ceftriaxone with other antibiotics or small molecules [66–68]. Upon hemolysis assay, the non-cytotoxicity of 4a-cot and 4b-cef (HC50 > 200 µg/mL; SI > 80) toward red blood cells is noteworthy.

Overall, this research work established the antibacterial activity of two heterocyclic azo dyes incorporating moieties of acetaminophen, and their combination with selected antibiotics against *Shigella* species that cause diarrhea. The mechanistic basis of the antibacterial action suggested a bactericidal orientation at 4 MIC, whereas the hemolysis test
showed the non-toxicity of test compounds and their combination with selected antibiotics (ceftriaxone and cotrimoxazole).

5. Limitations and Perspectives

This research sought to investigate the antibacterial activity of acetaminophen amalgamated diazobenzothiazoles against selected *Shigella* species. The combination of the active compounds (azo dyes 4a and 4b) with the least effective antibiotics (cotrimoxazole and ceftriaxone) showed a synergetic antibacterial effect with selectivity on human red blood cells. Moreover, compounds 4a and 4b were found to be potentially bactericidal vis-à-vis *S. flexneri* at 4XMIC. These compounds might provide chemical pharmacophores for repurposing acetaminophen to potentially active anti-diarrheal drugs. As it is important that the mechanism of action of these compounds be unveiled, kinetics of the bacterial growth were used to study their effect on the bacteria over time. However, major limitations of this work comprise cytotoxicity tests on various human cell lines (such as liver, kidney and intestinal epithelial cells, etc.) to identify the potential toxicity risks on vital organs in humans. In addition, more antibacterial tests should be extended to a larger set of other clinically relevant *Shigella* strains.

Furthermore, in-depth antimicrobial mechanisms of action and pharmacokinetics of compounds 4a and 4b should be investigated to warrant the successful utilization of these compounds in antibacterial drug discovery.

6. Conclusions

In this study, the antibacterial activity of acetaminophen 3 and its diazobenzothiazole derivatives 4 (a and b) was evaluated against four strains of *Shigella*, the pathogens responsible for infectious diarrhea. Acetaminophen 3, which is a well-known anti-inflammatory, analgesic and antipyretic drug used to treat pain and fever was used to synthesize a series of azo dyes incorporating moieties of acetaminophen 4. Upon in vitro anti-*Shigella* assays, these compounds exhibited antibacterial activity against *Shigella flexneri*. The combination of the azo dyes 4 (a and b) with cotrimoxazole and ceftriaxone showed a synergistic antibacterial effect and high selectivity vis-à-vis human red blood cells. These compounds were established through kinetics of bacterial growth to be potentially bactericidal and might serve as scaffolds for repurposing acetaminophen from its traditional use as an anti-inflammatory and antipyretic drug to potentially active anti-diarrheal agents against *Shigella*-causing diarrhea.


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the clinical isolate, *Shigella dysenteriae*. This research received support, material and equipment from the Yawunde—Bielefeld Bilateral Graduate School for Natural Products with Anti-parasite and Antibacterial Activity (YaBiNaPA).

**Conflicts of Interest:** The authors declare no conflicts of interest.

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