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Research Article
Synthesis and Antibacterial Assessment of Novel Azithromycin Derivatives as Potential Taste-Masking Prodrugs

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Abstract

Background and Objective: Azithromycin is a widely used macrolide antibiotic with broad-spectrum antimicrobial activity; however, its clinical acceptability is limited by poor patient compliance due to its bitter taste. Additionally, the growing threat of antimicrobial resistance necessitates the development of novel derivatives with improved therapeutic performance. This study aimed to synthesize and evaluate novel azithromycin derivatives with dual potential for taste masking and enhanced antibacterial activity. **Materials and Methods:** Five novel azithromycin derivatives were synthesized by reacting azithromycin with different functional linkers, including *N*-acetyl benzenesulfonyl chloride, *p*-nitrobenzoyl chloride, *p*-toluenesulfonyl chloride, 3,5-dinitrobenzoyl chloride and *p*-nitrobenzenesulfonyl chloride. The synthesized compounds were characterized using Fourier-Transform Infrared Spectroscopy (FT-IR), nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Prodrug properties were assessed through kinetic stability studies at physiological pH values (2.2, 5.5 and 7.4). Antibacterial activity was evaluated *in-vitro* using minimum inhibitory concentration (MIC) and Half-Maximal Inhibitory Concentration (IC_{50}) assays against clinically relevant bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. The bacterial strains used in this study were standard ATCC reference strains and were not derived from human or animal subjects. Therefore, ethical approval was not required. All experiments were conducted following standard microbiological laboratory practices. **Results:** All five azithromycin derivatives were successfully synthesized with satisfactory yields and confirmed structural characteristics. **Compounds 2**, **4** and **5** demonstrated favorable prodrug behavior by undergoing pH-dependent hydrolysis and regenerating the parent drug, indicating promising taste-masking potential. Furthermore, **compounds 1** and **3** exhibited superior antibacterial activity against *Pseudomonas aeruginosa* compared to the parent azithromycin. **Conclusion:** The synthesized azithromycin derivatives demonstrated promising potential as next-generation therapeutic agents with improved palatability and retained or enhanced antimicrobial efficacy. These findings support their prospective application in overcoming taste-related compliance issues while addressing antimicrobial resistance challenges.

Key words: Azithromycin, macrolide antibiotics, taste masking, prodrugs, antimicrobial activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Drugs possessing a macrocyclic lactone ring of twelve or more components are referred to as "macrolides"[1]. Erythromycin, the first and most popular macrolide, was brought to the market and utilized in medicine in 1952[2]. A common class of antibiotics is the 14-, 15- and 16-membered macrolides. They have superior tissue penetration and antibacterial action, primarily against atypical infections and Gram-positive cocci[1]. This well-known azalide antibiotic can be found in a range of bodily fluids and tissues and shares structural similarities with the macrolide family. The addition of a nitrogen atom to the macrolide aglycone ring distinguishes it chemically from erythromycin[3]. Compared to erythromycin, AZI has a longer half-life ($T_{1/2}$ = 68 hrs) and fewer gastrointestinal (GI) adverse effects. Patients benefit greatly from azithromycin's extended half-life, particularly children and older adults, due to the reduced frequency of administration needed and the increased patient adherence to treatment[4,5]. AZM prevents bacterial growth and protein production by reversibly cutting off the 50S bacterial ribosomal subunit[6]. The macrolides attach to the 50S ribosomal subunit, specifically targeting the 23S ribosomal RNA and several ribosomal proteins[7,8]. Since the late 1980s, azithromycin has been used extensively in medicine to treat a variety of bacterial illnesses. However, the number of pathogens becoming resistant to this antibiotic increases annually[9].

The widespread use of macrolide antibiotics over the past 50 years has resulted in bacterial resistance, which may even raise the risk of death from common pathogen infections in both rich and poor nations[10-12]. Various Macrolide Resistance Genes (MRGs) have been identified, resulting in resistance via various routes, including target changes generated by rRNA methylases carried in erm genes or macrolide inactivation, mediated by esterases such as those encoded by ere(A) or ere(B) genes and by phosphorylases like those found in the mph(A) and mph(B) genes. Furthermore, it has been found that transferable genes like msr(A), mef(A), or mef(B) encode macrolide-efflux pumps[13-19]. Azithromycin (AZM) is a macrolide antibiotic employed in the treatment of several bacterial illnesses. Due to its relatively high molecular weight, low solubility, slow rate of dissolution and partial intestinal absorption, the medication is reported to have limited oral bioavailability (37%)[20].

One of the main issues with treating kids is the unpleasant, bitter taste of oral antibiotics. The bitter taste of medications causes the pediatric population to throw up, which eventually results in less-than-ideal therapeutic value, grimacing and mental tension. Therefore, creating a palatable formulation especially to solve the problem is a difficult assignment for formulation scientists[21]. AZ is essentially insoluble in water but soluble at the pH of saliva, which easily solubilizes the drug content and exposes it to the tongue's taste buds, making it taste bitter[22-26]. Earlier studies on how to overcome the problem of taste-masking have utilized many different approaches, such as polymer coating using Eudragit, the use of microencapsulation, micronization, spheronization, ion exchange, the development of nanoparticles, granulation, taste potentiators, taste suppressants, sweeteners and flavors[27-30]. The addition of flavor and sweeteners is the most straightforward and popular method among these traditional methods for hiding the taste of a pediatric formulation; nonetheless, it is ineffective for extremely bitter medications like azithromycin (AZ), clarithromycin and vancomycin[21,31]. The method employed in this work is the production of prodrugs based on intramolecular mechanisms that mask the bitter taste of medications. The interconversion of a prodrug to its equivalent drug is catalyzed without the requirement for an enzyme. The type of linker attached to the drug determines the speed of drug release. The tongue's taste receptors interact with bitter-tasting molecules to produce a bitter sensation. The drug's bitterness may be reduced or eliminated by changing how it interacts with bitter taste receptors. This could be accomplished by suitably altering a bitter compound's size and structure to prevent its binding with the taste receptor cells found in the taste buds T2R[32,33].

MATERIALS AND METHODS

Study area and duration: The study area was situated in Arab American University, Jenin, Palestine. The duration of the study ranged from August 2025 to January 2026.

Reagents and instruments: Analytical-grade compounds are utilized. *N*-acetylsulfanilyl chloride, azithromycin, *p*-nitrobenzoyl chloride, 3,5-dinitrobenzoyl chloride, *p*-nitrobenzenesulfonyl chloride, *p*-toluene benzene sulfonyl chloride, sodium hydride (60%), ethyl acetate, hexane, THF, phosphate buffer (pH 7.5, 5.5, 4.4 and 2.2), HPLC-grade water, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, Mueller Hinton agar and Mueller Hinton broth. The American Type Culture Collection (ATCC) biological laboratories provided the testing microorganisms. *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 49619) were among the microorganisms. The bacterial strains used in this study were

standard ATCC reference strains and were not derived from human or animal subjects. Therefore, ethical approval was not required. All experiments were conducted following standard microbiological laboratory practices.

Melting points were determined using the Stuart melting point equipment (R00102618). A Fourier-transform infrared spectrophotometer (Shimadzu IR Spirit FT-IR spectrophotometer) was used to perform infrared spectroscopy. Nuclear magnetic resonance (NMR) spectra were acquired at the University of Jordan in Amman using a Bruker Avance 500 MHz spectrometer (Bruker, Switzerland). Mass spectra were acquired at Al-Ahliyya Amman University in Jordan utilizing an LC-MS/MS facility (Sciex 4500 QTRAP equipped with UPLC-PDA-MS/MS). A Waters ACQUITY Arc high-performance liquid chromatography device equipped with a C18 column was used to perform the chromatographic separation. Tuttnauer autoclave, Memmert incubator, microliter pipettes, 96-well plates and Thermo Fisher ELISA reader.

Synthesis and characterization

General synthesis of azithromycin derivatives: The derivatives were produced in a single pot in two stages. An optimal anhydrous solution was used to dissolve azithromycin, such as tetrahydrofuran (THF) and submerged in a bath with ice. Sodium hydride (NaH), a strong base, was added in portions to the mixture. The mixture was agitated until there was no more formation of hydrogen gas was formed, indicating the deprotonation of the hydroxyl groups on the macrolide and the production of related alkoxide anions. The Dropwise additions of the appropriate benzoyl or sulfonyl chloride were made to the reaction mixture. The reaction was allowed to proceed at ambient temperature for several hours and its thin-layer chromatography (TLC) was used to track progress. Subsequently, upon completion, the crude product underwent recrystallization Fig. 1.

Synthesis of *N*-acetylsulfanilyl azithromycin derivative (Compound 1): *N*-acetylsulfanilyl chloride (0.934 g, 3.99×10^{-3} moL, 3.5 eq) was subjected to nucleophilic acyl substitution. After dissolving azithromycin (1 g, 1.335×10^{-3} moL) in 50 mL of THF, sodium hydride (0.265 g, 0.011 moL, 5 eq) was gradually added while stirring constantly until all hydrogen bubbles were eliminated. After adding N-acetylsulfanilyl chloride while stirring constantly, the reaction mixture was left overnight to produce **compound 1**. After that, the product was collected, filtered and recrystallized. The mass of the product was 0.45 g (yield: 36%), m.p. = 203 205°C and Rf = 0.43 (hexane: ethyl acetate: 1:4). The IR (v in cm^{-1}): 3388 NH, 3200-3600 OH, 3020 aromatic C-H, 1644 amide C = O, 1520 aromatic C = C, 1347 S = O asymmetric stretch, 1145 S = O symmetric stretch and 856 (para-substituted aromatic ring). ^{1}H NMR (500 MHz, MeOD) δ in ppm: 8.5 (s, 1H, NH amide group), 7.64–7.87 (m, 4H, aromatic), 5.06 (s, 1H, α-H of ester in lactone ring), 4.86 (s, 1H, OCHO desosamine sugar), 4.56 (proton on carbon carrying the sulfonyl group), 3.62 (s, 3H, $1OCH_3$) and 3.38 (s, 6H, $2NCH_3$). MS (ESI^+) m/z: 947.2.

Synthesis of 4-nitrobenzoyl azithromycin derivative (Compound 2): 4-nitrobenzoyl chloride (0.877 g, 4.72×10^{-3} moL, 3 eq) was subjected to nucleophilic acyl substitution. After solubilizing azithromycin (1 g, 1.33×10^{-3} moL) in 50 mL of THF, sodium hydride (0.189 g, 0.0078 moL, 5 eq) was gradually added while stirring constantly until all hydrogen bubbles vanished. A 4-nitrobenzoyl chloride was then added while stirring continuously. **Compound 2** was produced by the reaction, which took place overnight. The organic product was filtered, collected and recrystallized after the product solution was extracted using ethyl acetate. Rf = 0.7 (hexane: Ethyl acetate: 1:4), m.p. = 181-186°C and the product's mass was 1 g (81% yield).

IR (v in cm^{-1}): 3200-3600 OH, 3020 (C-H aromatic), 1730 (Ester C = O), 1620 C = C aromatic, 1340 (NO_2 symmetric stretch) and 815 (para-substituted aromatic ring). ^{1}H NMR (500 MHz, acetone) δ in ppm: δ 8.24-8.25 (m, 4H, aromatic), 5.02 (s, 1H, α-H of ester in lactone ring), 4.81 (s, 1H, OCHO desosamine sugar), 4.62 (proton on carbon bearing the ester group), 3.40 (s, 3H, $1OCH_3$) and 2.57 (s, 6H, $2NCH_3$). MS ($ES0I^+$) m/z: 899.5.

Synthesis of 3,5-dinitrobenzoyl azithromycin derivative (Compound 3): 3,5-dinitrobenzoyl chloride (0.923 g, 4×10^{-3} moL, 3 eq) was subjected to nucleophilic acyl substitution. After dissolving azithromycin (1 g, 1.33×10^{-3} moL) in 50 mL of THF, sodium hydride (0.2935 g, 0.0122 moL, 5 eq) was gradually added while being continuously stirred until all hydrogen bubbles vanished. Then, with constant stirring, 3,5-dinitrobenzoyl chloride was added. **Compound 3** was produced when the reaction was carried out overnight. The organic product was filtered, collected and recrystallized after the product solution was extracted using ethyl acetate. The result had a mass of 0.575 g (yield 46%), a m.p. of 244-247°C and an Rf of 0.7 (hexane: Ethyl acetate: 1:4). IR (v in cm^{-1}): Broad absorption in 3200-3600 confirmed the presence of OH, 3060 (C-H aromatic), The ester carbonyl at 1711 (ester C = O), 1640 C = C aromatic and nitro group at 1380 (NO_2 symmetric stretch). ^{1}H NMR (500 MHz, DMF) δ in ppm: Aromatic protons

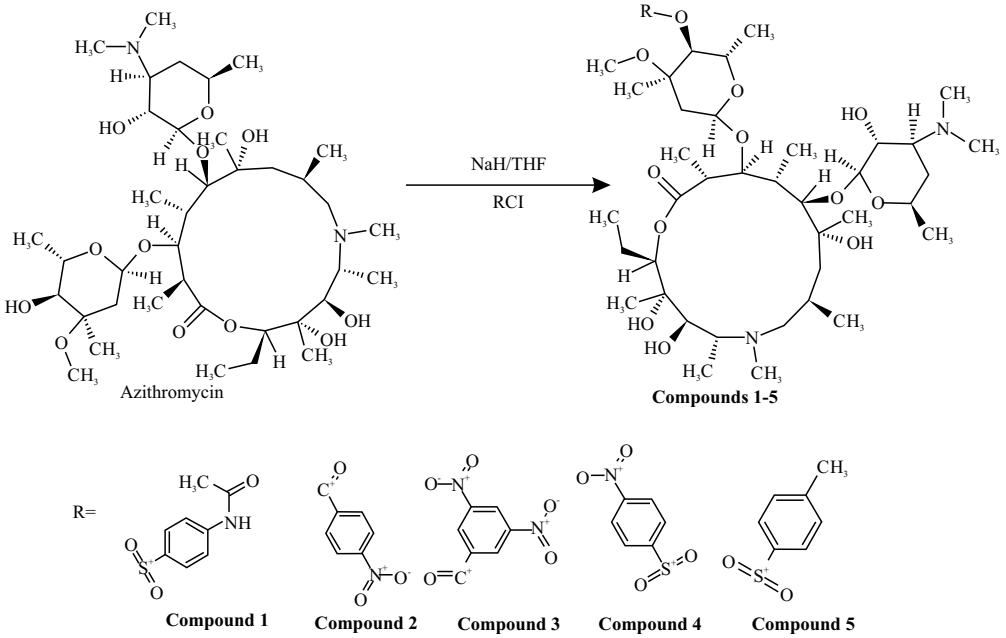


Fig. 1: Synthesis of azithromycin derivatives

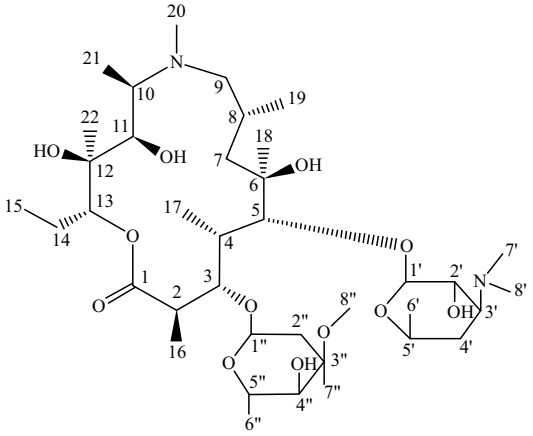


Fig. 2: Modifiable double prime 4-hydroxy group functional groups on the cladinose sugar ring of azithromycin[34]

at δ 9.26 (s, 2H, aromatic), 9.09 (s, 1H, aromatic), 5.17 (s, 1H, α-H of ester in lactone ring) and the α-proton of ester linkage in lactone ring at 5.15 (s, 1H, OCHO desosamine sugar), additional signals at 4.86 (proton on carbon bearing the ester group), methoxy groups were identified at 3.56 (s, 3H, $1OCH_3$), 3.10 (s, 6H, $2NCH_3$). MS (ESI^+) m/z: 944.

Synthesis of 4-nitrobenzenesulfonyl azithromycin derivative (Compound 4): 4-nitrobenzenesulfonyl chloride (0.887 g, 4×10^{-3} moL, 3 eq) was subjected to nucleophilic acyl substitution. After solubilizing azithromycin (1 g, 1.33×10^{-3} moL) in 50 mL of THF, sodium hydride (0.267 g, 0.011 moL, 5 eq) was added gradually while stirring constantly until all hydrogen bubbles vanished. **Compound 4** was then produced by adding 4-nitrobenzenesulfonyl chloride while continuously stirring the reaction mixture throughout the entire night. The organic product was filtered, collected and recrystallized after the product solution was extracted using ethyl acetate. Rf = 0.6 (hexane: Ethyl acetate: 1:4), m.p = 220-225°C and the product's mass was 0.99 g (yield 80%). IR (v in cm^{-1}): 3200-3600 OH, 3030 (C-H aromatic), 1621 (C = C aromatic), 1538 (N-O asymmetric stretch), 1380 (N-O symmetric stretch), 1166 (SO_2 symmetric stretch) and 796 (para-substituted aromatic ring). 1H NMR (500 MHz, acetone) δ in ppm: δ 8.24-8.05 (m, 4H, aromatic), 5.09 (s, ^{1}H, α-H of ester in lactone ring), 4.97 (s, 1H, OCHO desosamine sugar), 4.95 (proton on carbon bearing the sulfonyl group), 3.82 (s, 3H, $1OCH_3$) and 3.06 (s, 6H, $2NCH_3$). MS (ESI) m/z: 935.

Synthesis of 4-Toluenesulfonyl azithromycin derivative (Compound 5): 4-Toluenesulfonyl chloride (0.763 g, 4×10^{-3} moL, 3 eq) was subjected to nucleophilic acyl substitution. After dissolving 1 g of azithromycin (1.33×10^{-3} moL) in 50 mL of THF, sodium hydride (0.267 g, 0.011 moL, 5 eq) was gradually added with continuous stirring until there were no more hydrogen bubbles. **Compound 5** was then produced by adding 4-toluenesulfonyl chloride while continuously stirring the reaction mixture throughout the entire night. The organic product was filtered, collected and recrystallized after the product solution was extracted using ethyl acetate. The product had a mass of 0.5 g (yield 42%), m.p. of 184-187°C and Rf of 0.52 (1:4 hexane to ethyl acetate). IR (v in cm^{-1}): 3200-3600 OH, 3109 (Aromatic C-H stretch), 1525 (C = C aromatic), 1350 (asymmetric SO_2 stretch), 1150 (symmetric SO_2 stretch and 854 para-substituted aromatic rings). ^1H NMR (500 MHz, acetone) δ in ppm: δ (7.36-7.57) (m, 4H, aromatic), 5.09 (s, 1H, α-H of ester in lactone ring), 4.97 (s, 1H, OCHO desosamine sugar), 4.95 (proton on carbon bearing the sulfonyl group), 3.3 (s, 3H, $1OCH_3$), 3.04 (s, 6H, $2NCH_3$) and 2.41(s, 3H, CH_3 toluene). MS (ESI^+) m/z: 904.

Structure-activity analysis of azithromycin derivatives: Structure-activity analysis of azithromycin derivatives shows that one of the most promising avenues for creating improved analogs capable of boosting activity against pathogenic microorganisms, including macrolide-resistant variants, is to modify the double prime 4-hydroxy group of the cladinose residue (Fig. 2).

HPLC study of azithromycin prodrug hydrolysis at different ph levels

Azithromycin derivatives and azithromycin stock solution preparation: Using a 100 mL volumetric flask, 50 mg of azithromycin was dissolved in 50 mL of mobile phase (acetonitrile: Phosphate buffer (pH 7.5) in a 50:50 v/v ratio) to create a stock solution. The solution was sonicated for five minutes before the volume was adjusted with mobile phase to reach a concentration of 500 mg/L. The five azithromycin derivatives were prepared using the same method.

Making prodrug solutions at various pH levels: By dissolving 50 mg of each prodrug (1, 2, 3, 4 and 5) in 100 mL of solutions with pH values of 2.2, 5.5 and 7.4, five azithromycin prodrugs at a concentration of 500 ppm were produced. For a whole day, the samples were continuously shaken and incubated at 37°C. The retention period of the hydrolyzed azithromycin prodrugs was then measured by HPLC analysis of each sample.

Chromatographic conditions for solutions of azithromycin and azithromycin prodrugs: A C18 column (150×4.6 mm; 3.5 µm) with integrated UV detection at 215 nm is part of the HPLC apparatus. The mobile phase was made fresh, filtered and consisted of acetonitrile and phosphate buffer (pH 7.5) in a 50:50 v/v ratio, was administered at a flow rate of 1.2 mL/min and was sonicated before use. Each injection had a capacity of fifty microliters. For the analysis, the column temperature was maintained at 40°C and the runtime was approximately 9 min, which corresponds to the azithromycin retention time of 7.33 min.

Antibacterial activity: In accordance with the criteria of the Clinical and Laboratory Standards Institute (CLSI), the broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the substances tested. The IC_{50} values were obtained using Graphpad Prism version 8.0.2 (Graphpad Software, San Diego, CA, USA). Each concentration was evaluated in five duplicates and the data were entered into an XY table where X was the log (concentration) and Y was the percentage of inhibition. The inhibitor vs. response (three parameters) model was utilized to compute IC_{50} values using dose-response curves (nonlinear regression). The results are shown as Mean+S.D.

Preparation of prodrugs: The DMF was used to generate a 5 mg/mL stock concentration of each sample. An electric balance was used to weigh 0.05 g of each sample and then 10 mL of DMF were added to make 5 mg/mL. Mueller-Hinton broth (MHB) was serially diluted 1:2 to a final working concentration of 64 to 0.5 µg/mL. By measuring absorbance at 630 nm, the optical density was found.

Statistical analysis: All antimicrobial activity data, the Minimum Inhibitory Concentration (MIC) and Half-Maximal Inhibitory Concentration (IC_{50}), were analyzed using GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA). Non-linear regression was performed to create dose-response curves and IC_{50} values were determined from the regression curves. All measurements were done three times and the mean values with a standard deviation are presented.

The antimicrobial activities of the synthesized azithromycin derivatives were compared with the parent compound by comparing the calculated MIC and IC_{50}. Statistical significance was set at $p<0.05$.

RESULTS

Five novel azithromycin derivatives were successfully synthesized through a one-pot esterification reaction using various benzoyl and sulfonyl chloride derivatives. The synthesized compounds were purified by recrystallization and subsequently characterized using Fourier-Transform Infrared Spectroscopy (FT-IR), proton nuclear magnetic resonance (^1H NMR) and mass spectrometry (MS) to confirm their chemical structures. Following structural confirmation, the derivatives were evaluated for their hydrolytic behavior under simulated gastrointestinal and physiological conditions (pH 2.2, 5.5 and 7.4) using High-Performance Liquid Chromatography (HPLC). Their antibacterial activities were then assessed against representative Gram-positive and Gram-negative bacterial strains to determine whether the structural modifications influenced antimicrobial efficacy.

The obtained results demonstrated considerable differences among the synthesized derivatives regarding both hydrolysis and biological activity. While several compounds exhibited limited or partial conversion to the parent azithromycin molecule, one derivative in **compound 5** showed complete hydrolysis under intestinal and physiological conditions, indicating its potential as an effective taste-masking prodrug. In addition, antibacterial testing revealed that the structural modifications produced distinct activity profiles, with some derivatives displaying enhanced potency against selected bacterial species compared with azithromycin. These findings provide valuable insight into the relationship between chemical structure, hydrolytic stability and antibacterial performance, supporting further development of selected derivatives for pharmaceutical applications.

Azithromycin characterization study: All of the prepared substances were analyzed by IR, 1H and MS analysis. The raw data for all produced compounds collected are accessible in the supplementary materials. The primary features of IR spectroscopy include C = O bonds for ester carbonyl around 1735-1750 cm^{-1}, ester bonds (benzoyl esters), S = O stretch, 1150-1360 cm^{-1} (asymmetric and symmetric S = O stretch) and sulfonate esters. The aromatic C = C stretching vibration of sulfonyl and benzoyl groups at 1500-1600 cm^{-1}. Nitro group stretching vibrations on aromatic groups at 1520-1550 cm^{-1} (asymmetric NO_2 stretch) and 1340-1380 cm^{-1} (symmetric NO_2 stretch) of derivatives with nitro substitutions. Characteristic Signals of ^1H NMR: Macrolide proton backbone: δ 0.8-4.5 ppm (complex multiplets for azithromycin structure), ester (sulfonyl)-linked protons: δ 4.5-6.0 ppm (proton on carbon near the ester group in lactone ring) and aromatic protons chemical shift: δ 7.2-8.2 ppm. Molecular Mass Spectrometry: The derivative molecular weight is represented by the ion peaks $[M+H]^+$.

Azithromycin derivatives: Hydrolysis study: The results are organized to provide a detailed analysis of the stability and degradation trend of derivatives at different pH levels. High-Performance Liquid Chromatography (HPLC) was used to monitor the hydrolysis process and identify any degraded products within a day. The retention times of azithromycin and its five derivatives were determined by HPLC analysis (Table 1).

Hydrolysis at pH 2.2 (gastric conditions): Compounds 2, 4 and **5** showed the most promising hydrolysis properties, partially returning to the initial azithromycin structure. The parent antibiotic was released after these prodrugs underwent controlled hydrolysis, confirming that the prodrug design was successful in masking taste without losing therapeutic efficacy (Fig. 3-5).

The parent azithromycin peaks at retention time 7.248 min and a secondary peak at retention times 3.084 and 8.296 min, corresponding to other degradation products shown in Fig. 3.

The parent azithromycin peaks at retention time 7.327 min and a secondary peak at retention times 3.070 and 5.709 min, corresponding to other degradation products shown in Fig. 4.

The parent azithromycin peaks at retention time 7.399 min and a secondary peak at retention times 3.082, 6.438 and 8.053 min, corresponding to other degradation products shown in Fig. 5.

Compounds 1 and **3** showed a different hydrolysis profile as a result of partial or complete dissociation, which prevented the original azithromycin molecule from forming. Instead, these compounds produced breakdown products with varying retention times, indicating alternative degradation routes.

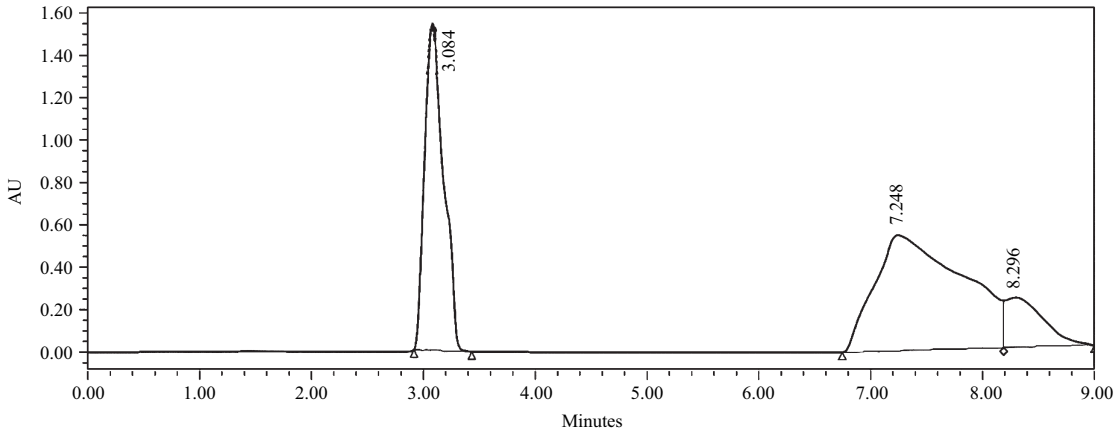


Fig. 3: **Compound 2**'s representative HPLC chromatogram at PH 2.2

AU stands for Absorbance Units

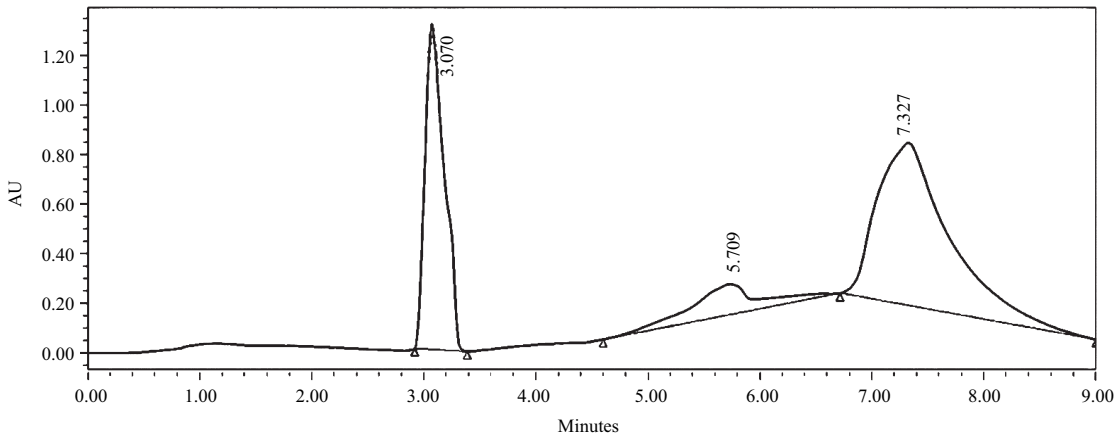


Fig. 4: **Compound 4**'s representative HPLC chromatogram at PH 2.2

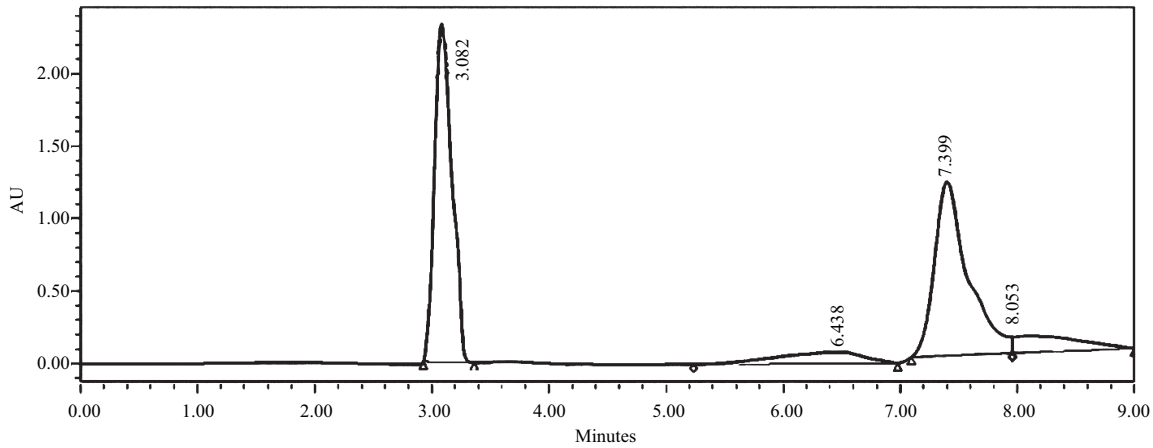


Fig. 5: **Compound 5**'s representative HPLC chromatogram at PH 2.2

Table 1: Prepared prodrug retention times: Display each prodrug examined on its own (without adjusting pH)

Compounds	Retention times (minutes)
Azithromycin	7.33
Compound 1	3.662
Compound 2	3.797
Compound 3	3.801
Compound 4	1.265
Compound 5	2.934

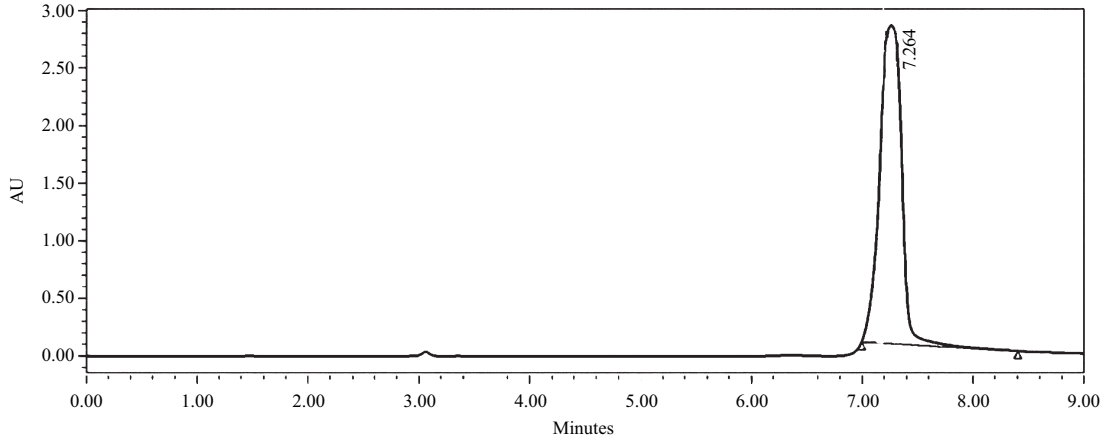


Fig. 6: **Compound 5**'s representative HPLC chromatogram at PH 5.5

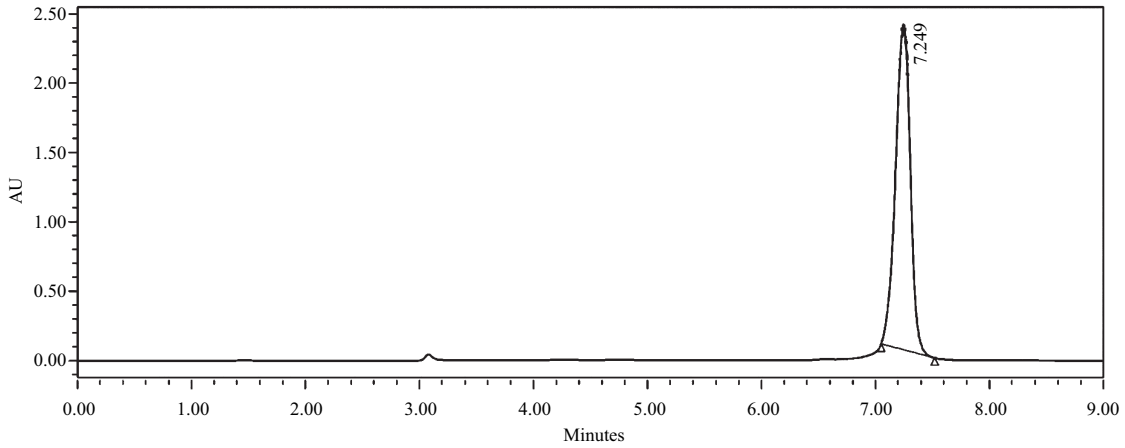


Fig. 7: **Compound 5**'s representative HPLC chromatogram at pH 7.4

Table 2: Complete hydrolysis results for compounds hydrolyzed to parent azithromycin at pH 2.2, 5.5 and 7.4

Compounds	pH	Remaining prodrug (%)	Azithromycin formed (%)	Other products (%)
Compound 2	2.2	0	55.49	44.51
Compound 4	2.2	0	58.29	41.71
Compound 5	2.2	42.92	42.31	14.77
Compound 5	5.5	0	100	0
Compound 5	7.4	0	100	0

Hydrolysis at pH 5.5 (duodenal transition zone): The most favorable hydrolysis was shown by **compound 5**, which was successfully converted back to the original azithromycin molecule (Fig. 6). The parent antibiotic was recovered after this prodrug underwent controlled hydrolysis, demonstrating the effective prodrug design that masks the taste while maintaining therapeutic activity.

The single sharp parent azithromycin peak at retention time 7.264 minutes represents the released azithromycin after complete hydrolysis.

Particularly at and below pH 5.5, the majority of prodrugs that were evaluated (**compounds 1**, **2**, **3** and **4**) showed partial or complete hydrolysis and failed to return the original azithromycin molecule. Instead, the compounds exhibited unexpected degradation pathways, resulting in various degradation products with differing retention times.

Hydrolysis behavior at pH 7.4 (physiological/blood pH): At pH 7.4, **compound 5** obtained its maximal conversion to azithromycin, demonstrating its efficacy as a prodrug (Fig. 7). The single sharp parent azithromycin peak at retention time 7.249 min represents the released azithromycin after complete hydrolysis.

Compounds 1, **2**, **3** and **4** exhibit high degradation but with no azithromycin formation. The complete hydrolysis percentage of compounds that hydrolyze to the azithromycin parent compound is presented in Table 2.

Table 3: Azithromycin derivatives' minimum inhibitory concentrations against four test bacteria

Compound	*Escherichia coli* (MIC, µg/mL)	*Pseudomonas aeruginosa* (MIC, µg/mL)	*Streptococcus pneumoniae* (MIC, µg/mL)	*Staphylococcus aureus* (MIC, µg/mL)
Compound 1	4	8	4	4
Compound 2	4	4	4	4
Compound 3	4	8	4	4
Compound 4	4	8	8	8
Compound 5	4	8	8	8
Azithromycin	1	>16	2	1

Table 4: Azithromycin derivatives' IC_{50} values versus test microorganisms

Compound	*Escherichia coli* (IC_{50}, µg/mL)	*Pseudomonas aeruginosa* (IC_{50}, µg/mL)	*Streptococcus pneumoniae* (IC_{50}, µg/mL)	*Staphylococcus aureus* (IC_{50}, µg/mL)
Compound 1	3.42±0.18	1.65±0.31	1.80±0.25	15.66±0.44
Compound 2	1.71±0.10	0.81±0.11	1.20±0.21	6.11±0.57
Compound 3	18.97±0.36	2.79±0.16	0.60±0.30	1.25±0.24
Compound 4	0.56±0.29	0.10±0.19	2.85±0.21	61.56±0.45
Compound 5	1.63±0.13	11.71±0.25	1.80±0.37	9.83±0.36
Azithromycin	0.34±0.08	4.19±0.54	0.41±0.14	0.29±0.12

The mean±standard deviation is used to report the values. R^2 values were more than 0.95 for every dose-response curve

Table 5: Azithromycin derivatives' IC_{50} values against *Escherichia coli* were sorted from highest to lowest activity

Compound	*Escherichia coli* (IC_{50}, µg/mL)
Azithromycin	0.34±0.08
Compound 4	0.56±0.29
Compound 5	1.63±0.13
Compound 2	1.71±0.10
Compound 1	3.42±0.18
Compound 3	18.97±0.36

Biological activities: A panel of four bacterial strains two Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) was used to test the antibacterial activity of the five produced *compounds in vitro* (Table 3). Table 4 shows these substances' IC_{50} values and minimal inhibitory concentrations. The azithromycin derivatives exhibited different degrees of antibacterial activity, both in terms of MIC and IC_{50} values, against the four test bacteria, with distinct structure-activity relationships. All t he derivatives were significantly more active than the parent compound against *E. coli,* with MIC values being fourfold higher in all the derivatives; **compound 4** was the most potent (0.56±0.29 µg/mL) and **compound 3** was the least potent (18.97±0.36 µg/mL). **Compound 4** showed excellent IC_{50} potency (0.10±0.19 µg/mL) even though the MIC value was 8 µg/mL, which is 41.9-fold lower than the parental drug against *P. aeruginosa.* **Compounds 1-3** had MIC values of 4 µg/mL against *S. pneumoniae,* with the lowest IC_{50} of 0.60±0.30 µg/mL for **compound 3**. For *S. aureus*, the MIC for **compounds 1-3** remained 4 µg/mL, but **compound 3** showed the best IC_{50} (1.25±0.24 µg/mL and **compound 4** showed severely compromised activity (IC_{50} = 61.56±0.45 µg/mL.

Comparison with azithromycin standard: The IC_{50} values against *E. coli* are shown in Table 5. Azithromycin had the highest efficacy among the investigated drugs, with an IC_{50} of 0.3379 µg/mL. **Compound 4** showed encouraging activity with an IC_{50} of 0.5639 µg/mL (Fig. 8), which is similar to azithromycin.

The compounds' activity against *P. aeruginosa* is displayed in Table 6. It's interesting to note that **compound 4** has an IC_{50} value of 0.1 µg/mL, indicating remarkable potency (Fig. 9). Compared to azithromycin (IC_{50} = 4.188 ug/mL), this was far superior.

As for testing the compounds against *Streptococcus pneumoniae and Staphylococcus aureus*, the following tables (Table 7, 8) show the results.

The final example of an efficient prodrug in this work is **compound 5**, which is the *p*-tolylsulfonyl chloride analogue of azithromycin. It would be successful in releasing the active medication in the small intestine and the systemic circulation because it was completely hydrolyzed to the parent azithromycin at pH values of 5.5 and 7.4.

This, along with the covalent alteration's likely reduced bitterness, makes it a suitable option for an azithromycin formulation that masks flavor. Its potential to provide a quick start of action a desirable feature for the treatment of acute

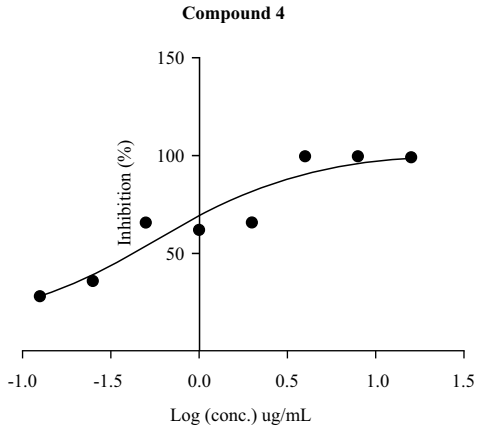


Fig. 8: **Compound 4**'s IC_{50} on *Escherichia coli*

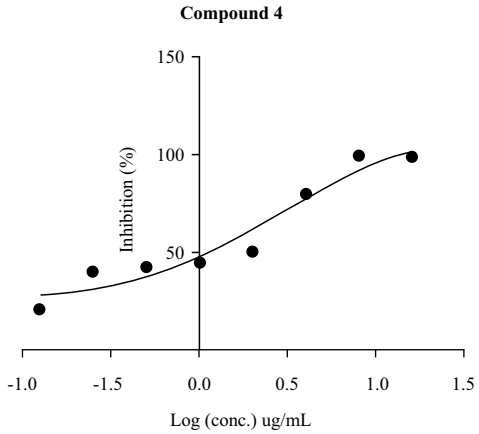


Fig. 9: **Compound 4**'s IC_{50} on *Pseudomonas aeruginosa*

Table 6: Azithromycin derivatives' IC_{50} values against *Pseudomonas aeruginosa* were sorted from highest to lowest activity

Compound	*Pseudomonas aeruginosa* (IC_{50}, μg/mL)
Compound 4	0.10±0.02
Compound 2	0.81±0.11
Compound 1	1.65±0.31
Compound 3	2.79±0.16
Azithromycin	4.19±0.54
Compound 5	11.71±0.25

Table 7: Azithromycin derivatives' IC_{50} values against *Streptococcus pneumoniae* were sorted from highest to lowest activity

Compound	*Streptococcus pneumoniae* (IC_{50}, μg/mL)
Azithromycin	0.41±0.14
Compound 3	0.60±0.30
Compound 2	1.20±0.21
Compound 5	1.80±0.37
Compound 1	1.80±0.25
Compound 4	2.85±0.21

infections is further suggested by its capacity to partially hydrolyze at pH 2.2. After dissociation, **compound 5** will have the same antimicrobial activity as parent azithromycin. It was found that at pH 2.2, **compounds 2** and **4** degraded to azithromycin, indicating that they could potentially overcome the bitter taste with *in vivo* regeneration of azithromycin. **Compounds 1** and **3** were found to be more effective against *Pseudomonas aeruginosa* than azithromycin. It is important to note that they do not break down to azithromycin, but instead, they produce different products. This implies that these compounds may become potential novel therapeutic agents and may possibly do so by a different mechanism of action or through an analogous macrolide mechanism.

Table 8: Azithromycin derivatives' IC_{50} values against *Staphylococcus aureus* were sorted from highest to lowest activity

Compound	*Staphylococcus aureus* (IC_{50}, μg/mL)
Azithromycin	0.29±0.01
Compound 3	1.25±0.24
Compound 2	6.11±0.57
Compound 5	9.83±0.36
Compound 1	15.66±0.44
Compound 4	61.56±0.45

DISCUSSION

This current study was able to successfully synthesize a series of new azithromycin derivatives designed as potential taste-masking prodrugs that had the same or enhanced antibacterial properties as the parent compound. Azithromycin is highly prescribed for respiratory, skin and soft tissue infections and has an extremely bitter taste, which makes it difficult for patients, especially children and the elderly, to adhere to treatment. The main objective of the present work was the application of a "prodrug" strategy to temporarily modify the hydroxyl functionalities of azithromycin via esterification with benzoyl and sulfonyl groups to mask the bitter taste and then rehydrate the active drug after delivery. The obtained results indicate that chemical modification was effective in changing the hydrolytic properties as well as antibacterial activity of azithromycin derivatives, with significant differences in the biological activity depending on the type of substituent.

The overall results of the present work indicate that azithromycin can be successfully modified in a rational manner to create a series of derivatives with varying hydrolytic and antibacterial properties. Of all the compounds synthesized, **compound 5** proved to be the most promising prodrug as it not only had the potential for therapeutic activity but also reconverted to azithromycin in a physiologically relevant fashion. **Compounds 1-4**, however, exhibited novel antibacterial properties, especially against *Pseudomonas aeruginosa* and some of the derivatives are potentially promising lead compounds for the synthesis of new macrolide antibiotics. Altogether, these findings indicate the potential for additional optimization and *in vivo* testing of azithromycin derivatives as prodrugs and new antimicrobial drugs.

We can summarize the findings of the present study and compare them with those reported by previous researchers as follows: The hydrolysis reactions of the azithromycin derivatives 1-5 clearly show that they exhibit a structure-activity relationship, depending on the electronic properties of the aromatic substituents. The findings are similar to those of Stella and Nti-Addae who have found that structural changes of macrolides can have a profound influence on their chemical stability and hydrolysis rates, especially in terms of the electron-donating and electron-withdrawing substituents, **compounds 2** and **3** with nitro benzoyl groups exhibited hydrolysis profiles that were changed relative to the strong electron withdrawing character of the nitro group that increases the bond polarization and also has a significant impact on the hydrolytic stability[27,35]. The chemistry and testing of the azithromycin prodrug derivatives in the present study are similar to those previously reported for taste-masked macrolide formulations. The synthesis and identification of **compound 5** as a potential prodrug that hydrolyzes at pH 2.2 but remains stable at pH 7.4 are in line with the prodrug design principle proposed by Amin *et al.*[21]. The successful chemical modification of hydroxyl groups on the cladinose sugar ring confirms the feasibility of esterification of these groups, thus corroborating previous reports on macrolide derivatization. In addition, the observation that **compounds 2**, **4** and **5** also provide much comparative information on linker-dependent hydrolysis kinetics that has not been well documented in previous macrolide prodrug literature. Multiple linkers were evaluated comprehensively and comparative data on their structure-activity relationships were provided, addressing an important gap in the literature for the systematic evaluation of linkers for taste-masking applications. It is important to note this pH-dependent hydrolysis profile in relation to the results obtained by Tung *et al.*[4] and Mashaqbeh *et al.*[27], who found that the successful taste-masked prodrugs must be able to release the core drug at neutral pH and be stable in acidic media. Data from the hydrolysis show not only confirmation, but enhancement of the theoretical predictions for ideal prodrug behavior. The present study provides important insights into the design of novel azithromycin derivatives as potential taste-masking prodrugs while simultaneously evaluating their hydrolytic behavior and antibacterial properties. The results demonstrate that strategic chemical modification of azithromycin can markedly influence both its physicochemical characteristics and biological performance, highlighting the importance of rational prodrug design in improving pharmaceutical formulations. In general, the findings contribute to the growing body of research focused on enhancing patient compliance through taste masking without compromising therapeutic efficacy. In addition, the

modifications in antibacterial activity of some of the derivatives indicate that the structures of these compounds could influence bacterial resistance mechanisms, interactions with the target and/or membrane permeability. In addition to establishing the feasibility of the development of taste-masking azithromycin prodrugs, it is noteworthy to point out the potential of chemical derivatization as a tool to identify macrolide-based antibacterial products with new or altered biological properties. There are a few shortcomings that should also be noted. Hydrolysis experiments were conducted under controlled *in vitro* conditions of pH, but the *in vivo* complexity of the gastrointestinal tract and systemic metabolism cannot be fully reproduced. The *in vivo* effects of enzyme hydrolysis, plasma esterase, intestinal transporters and hepatic metabolism on the activation of prodrugs may vary. Likewise, only four representative bacterial species were tested for antibacterial activity. To get a more complete picture of potential therapeutic value, broader antimicrobial screening, including multidrug-resistant clinical isolates, should be performed. Prior to any of these derivatives being considered for clinical development, toxicological evaluation and pharmacokinetic studies will be required. These studies will give a more complete evaluation of their potential for clinical development.

CONCLUSION

This work shows that the restricted antibacterial effectiveness and poor palatability of important macrolide antibiotics can be concurrently addressed by rational prodrug and analogue design, led by pH-sensitive linkers and electronic design. We showed that a hydrolysis behavior could be selectively controlled over the gastrointestinal pH range by introducing sulfonyl and benzoyl moieties to azithromycin. **Compound 5** (azithromycin p-toluene sulfonyl chloride) turned out to be an excellent taste-masked prodrug that is partially hydrolyzed at the pH found in the stomach and completely converted to the drug at pH in the intestine and at physiological conditions, supporting both rapid onset and full systemic availability. Interestingly, the **compounds 2** and **4** were observed to degrade into azithromycin under acidic conditions (pH 2.2), indicating that they could reduce the bitter taste as well as allow regeneration of the parent drug *in vivo*, whereas **compounds 1** and **3** had higher antibacterial activity relative to azithromycin in *Pseudomonas aeruginosa*, without degrading to azithromycin. All these results point to the fact that these derivatives can become a promising candidate for the development of a better antibacterial agent with improved pharmacological and taste characteristics.

SIGNIFICANCE STATEMENT

This study presents novel azithromycin derivatives designed to address two important limitations of azithromycin therapy: Its pronounced bitterness and the increasing challenge of antimicrobial resistance. The synthesized derivatives demonstrated favorable pH-dependent hydrolysis, supporting their potential as taste-masking prodrugs, while selected compounds showed enhanced antibacterial activity against *Pseudomonas aeruginosa* compared with the parent drug. These findings provide a promising foundation for developing more palatable azithromycin formulations without compromising antimicrobial efficacy and may contribute to improved patient acceptability and therapeutic outcomes. Further pharmaceutical and *in vivo* studies are warranted to confirm their safety, pharmacokinetic properties, taste-masking performance and clinical potential.

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