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Novel zafirlukast derivatives exhibit selective antibacterial activity against *Porphyromonas gingivalis*†

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Periodontal disease is an oral chronic immune-inflammatory disease highly prevalent worldwide that is initiated by specific oral bacterial species leading to local and systemic effects. The development of new preventive/therapeutic strategies to specifically target oral periodontopathogens without perturbing oral microbiome species normally colonizing the oral cavity is needed. The fast and affordable strategy of repositioning of already FDA-approved drugs can be an answer to the development of novel treatments against periodontal pathogens such as *Porphyromonas gingivalis*. Herein, we report the synthesis and antibacterial activity of novel zafirlukast derivatives, their bactericidal effect, and their cytotoxicity against oral epithelial cell lines. Many of these derivatives exhibited superior antibacterial activity against *P. gingivalis* compared to the parent drug zafirlukast. The most promising compounds were found to be selective against *P. gingivalis* and they were bactericidal in their activity. Finally, we demonstrated that these potent derivatives of zafirlukast provided a better safety profile against oral epithelial cells compared to zafirlukast.

Periodontal disease is a chronic inflammatory disease that is triggered by oral pathogenic bacteria (*e.g.*, *Porphyromonas gingivalis*) and leads to tooth loss if untreated. This disease affects half of the adult population in the United States and its prevalence significantly increases with aging.[1,2] In addition to the oral local sequelae, periodontal disease has also been associated as a risk factor for diabetes, cardiovascular diseases, stroke, as well as arthritis.[3–5] *P. gingivalis* is a Gram-negative bacterium that is associated with periodontal disease.[6–9] Evidence indicates that *P. gingivalis* can invade oral epithelial cells and modulate innate responses that lead to oral dysbiosis and unresolved chronic inflammation.[10–13] Although it has been suggested that local and systemic antibiotics can be used as adjunctive therapy for controlling periodontal disease,[14–16] there are no clear understanding and/or guidelines when it comes to selecting an antibiotic regimen for targeting specific periodontopathogens, without perturbing the normal oral commensal bacteria.[17,18] This pitfall can result in poor efficacy against the targeted pathogens, which can be either resistant or poorly susceptible against the antibiotic(s) selected.[19] In addition, the extensive use of antibiotics can result in development of drug-resistant bacteria, which would impact the effectiveness of the treatment. Thus, it is paramount that development of novel antibacterial agents for the treatment of periodontal disease becomes a priority.

A strategy currently used for the fast and affordable drug development that has been demonstrated to be successful involves the repositioning of existing drugs for new application. Compared to traditional methods, drug repositioning could provide a more systematic and significantly less expensive approach for developing treatments for complex diseases.[19–21] Drug repositioning can also be used as a strategy for hard to manage bacterial infections.[22] Novel antibacterial activity has been reported for existing FDA-approved anticancer, antifungal, anthelmintic, and anti-inflammatory drugs.[23–27] Zafirlukast is an FDA-approved drug used for the effective inhibition of airway inflammation in the case of asthma treatment.[28,29] Recently published work emphasized the use of zafirlukast as a treatment option for *Mycobacterium tuberculosis* and West Nile virus infections.[30,31] The antibacterial

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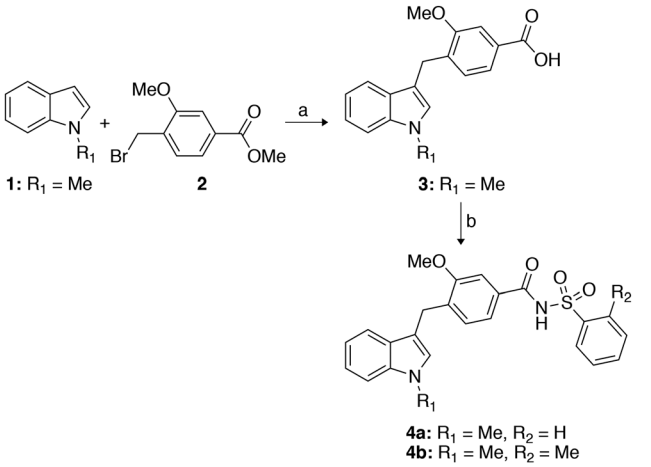
† Electronic supplementary information (ESI) available: Details of all experimental procedures for (i) the synthesis and characterization of all compounds and intermediates generated in this study, (ii) the effect of zafirlukast derivatives on bacterial viability, (iii) the bactericidal effect of zafirlukast derivatives, and (iv) the cytotoxic effect of zafirlukast derivatives on oral epithelial cells. All ^{1}H and ^{13}C NMR spectra as well as HPLC traces for the molecules generated (Fig. S1–S56). Tables showing the data used to create Fig. 5 (Table S1), Fig. 6 (Table S2), and Fig. 7 (Table S3). See DOI: 10.1039/c9md00074g

activity of zafirlukast against two oral pathogens, *P. gingivalis* and *Streptococcus mutans*, was also reported in recent studies.[32] Inspired by these findings, we postulated that chemically modifying zafirlukast could potentially lead to better agents for treating oral infections compared to this parent drug scaffold (Fig. 1). In zafirlukast, both a cyclopentyl carbamate attached to an *N*-methylindole and an arylsulfonamide scaffold are linked *via* a decorated benzoyl ring, which offers us an ideal opportunity for some structure–activity relationship (SAR) studies. Herein, we report the synthesis of eleven zafirlukast derivatives where we made modifications in the indole (*e.g.*, removal or replacement of the cyclopentyl carbamate by a nitro group and removal of the methyl group from the indole amine), the arylsulfonamide (*e.g.*, removal of the methyl substituent), and the benzoyl ring (*e.g.*, change in its substituents and substitution patterns). We also report the antibacterial activity of these zafirlukast derivatives against *P. gingivalis* and various other oral bacteria. In addition, we explore the cytotoxic effect of these zafirlukast derivatives against human oral epithelial cells.

Results and discussion

Design and synthesis of novel zafirlukast derivatives

Several synthetic methods were reported for either linear or convergent synthesis of zafirlukast.[29,30,33–39] Here, the concise and linear synthesis of zafirlukast derivatives involved C–C bond formation between various substituted indoles and benzoyl analogues followed by a subsequent sulfamidation step. The initial synthesis centered on creating zafirlukast derivatives **4a** and **4b** with variations in the arylsulfonamide domain as well as the elimination of the cyclopentyl carbamate of the indole of the parent molecule (Scheme 1). The alkylation of 1-methylindole in the presence of silver oxide



Scheme 1 The synthesis of zafirlukast derivatives **4a** and **4b**. Reaction conditions: (a) Ag_2O, dioxane, 60 °C, MeOH : THF : H_2O/5 : 1 : 1, KOH, room temperature, 29%; (b) arylsulfonamide, EDC·HCl, DMAP, CH_2Cl_2, room temperature, 59–84%.

followed by ester hydrolysis resulted in compound **3** in 29% yield. The coupling of acid **3** with both benzenesulfonamide and *o*-toluenesulfonamide generated target compounds **4a** and **4b** in 84 and 59% yields, respectively. The derivatives devoid of a methoxy group substituent on the benzoyl ring were synthesized according to Scheme 2. The condensation reaction between methyl 4-formylbenzoate with both indole and 1-methylindole resulted in compounds **7** and **8** in 23% and 56% yields, respectively. The hydrolysis of these compounds followed by their coupling with both benzenesulfonamide and *o*-toluenesulfonamide generated derivatives **11a**, **11b**, and **12a** in 58–100% yields. The synthesis of the final set of

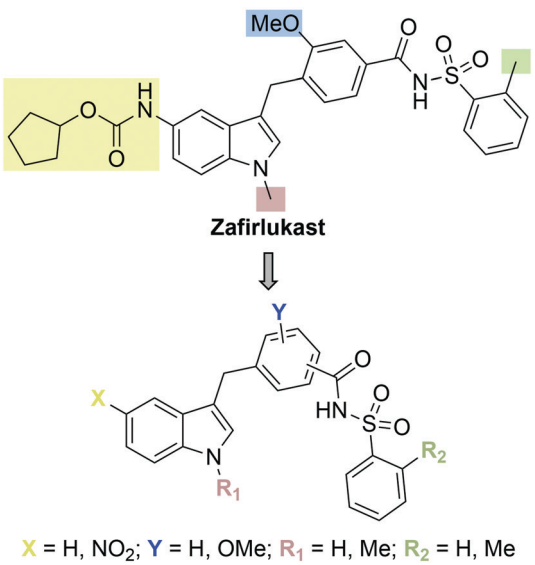
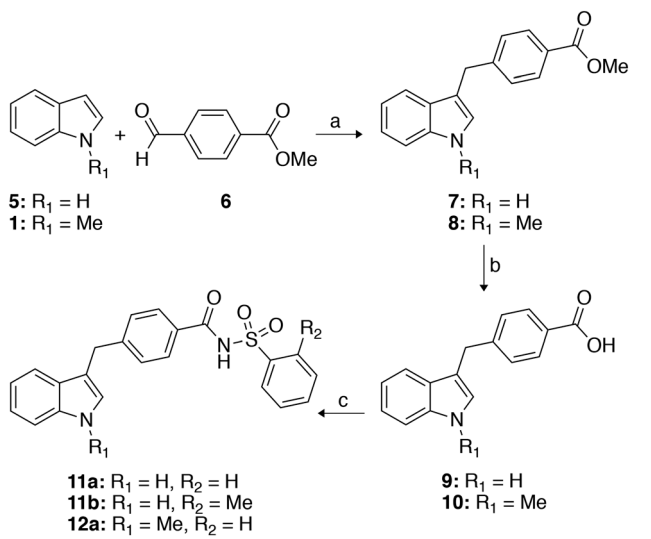


Fig. 1 Chemical structure of zafirlukast and its derivatives synthesized herein.



Scheme 2 The synthesis of zafirlukast derivatives **11a**, **11b**, and **12a**. Reaction conditions: (a) Et_3SiH, TFA, CH_2Cl_2, 0 °C to room temperature, 23–56%; (b) MeOH : THF : H_2O/5 : 1 : 1, KOH, room temperature, 83–84%; (c) arylsulfonamide, EDC·HCl, DMAP, CH_2Cl_2, room temperature, 58%-quant.

derivatives with modifications in the indole, benzoyl, and arylsulfonamide regions is depicted in Scheme 3. The condensation reaction between indole, 1-methylindole, and 1-methyl-5-nitroindole with compound **14**, a commercially available molecule, generated compounds **15**, **16**, and **17** in 43–89% yields. Finally, hydrolysis and sulfamidation yielded derivatives **21a**, **21b**, **22a**, **22b**, **23a**, and **23b** in 20–95% yields.

In vitro antibacterial testing

The antibacterial activity of the zafirlukast derivatives **4a**, **4b**, **11a**, **11b**, **12a**, **21a**, **21b**, **22a**, **22b**, **23a**, and **23b** was first evaluated against *P. gingivalis* 381 at concentrations 1, 10, and 100 μM (Fig. 2). Commercially available drugs such as zafirlukast (at 25 and 50 μM) and tetracycline (at 2.25 μM) were used as positive controls for comparison. By a rapid survey of the data presented in Fig. 2, we could conclude that all the zafirlukast derivatives exhibited 100% growth inhibition with the exception of compounds **4a** and **4b** at the highest concentrations tested. For compounds **4a** and **4b** we observed slightly more than 60% growth inhibition at 100 μM. At 10 μM, compounds **11a**, **11b**, **21a**, and **21b** displayed approximately 40%, 90%, 40%, and 80% growth inhibition, respectively. For the most active compounds **12a**, **22a**, **22b**, **23a**, and **23b**, we observed 90–100% growth inhibition at 10 μM. More importantly, the active compounds **12a**, **22a**, **22b**, **23a**, and **23b** exhibited either comparable or, in most of the cases, enhanced antibacterial activity against *P. gingivalis* when compared to the control drugs zafirlukast and tetracycline. Compounds **22b**, **23a**, and **23b** even displayed growth inhibition (~15–40%) at 1 μM. These three compounds were therefore selected for further studies.

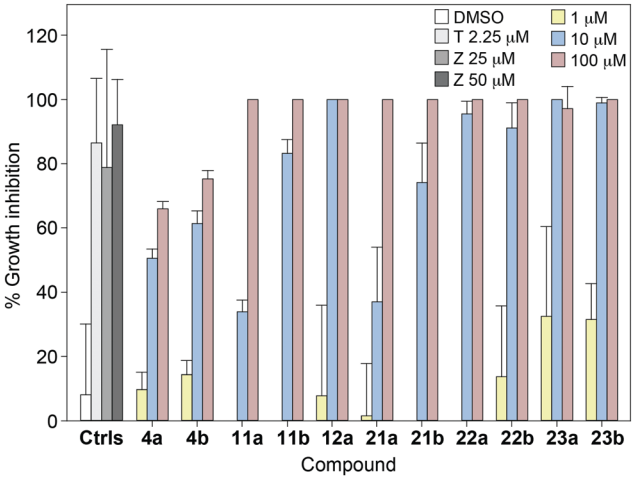
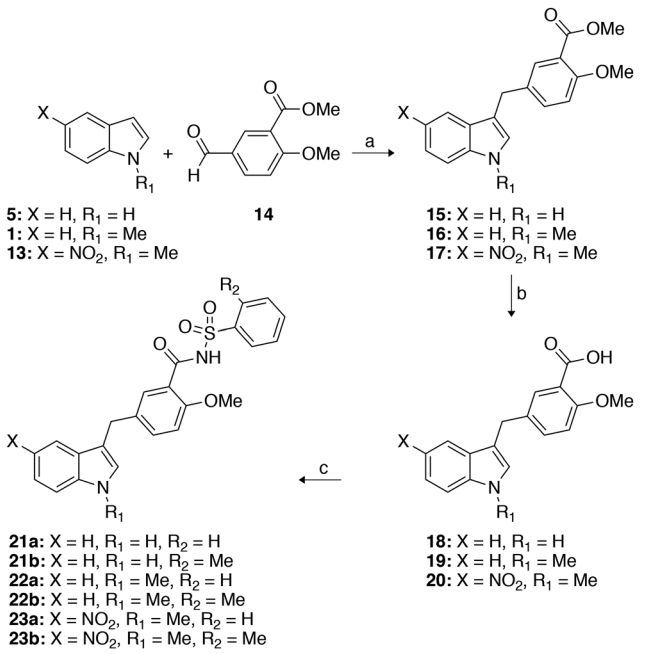


Fig. 2 Screening for the antimicrobial effect of the zafirlukast derivatives against *P. gingivalis*. *P. gingivalis* (10^6 cells) exponentially growing in appropriate medium and under anaerobic conditions was exposed to zafirlukast (Z) derivatives **4a**, **4b**, **11a**, **11b**, **12a**, **21a**, **21b**, **22a**, **22b**, **23a**, and **23b** (1, 10, and 100 μM) for 24 h. Bacteria incubated with tetracycline (T, 2.25 μM equivalent to 1 μg mL^{-1}) or zafirlukast (Z, 25 and 50 μM) were used as positive controls. Bacteria incubated with DMSO were used as a negative control. Data represent the mean inhibitory effect *versus* bacteria growing only in medium from 6 replicates per condition determined by a colorimetric WST-1 assay.



Scheme 3 The synthesis of zafirlukast derivatives **21a**, **21b**, **22a**, **22b**, **23a**, and **23b**. Reaction conditions: (a) Et_3SiH, TFA, CH_2Cl_2, 0 °C to room temperature, 43–89%; (b) MeOH : THF : H_2O/5 : 1 : 1, KOH, 65 °C, 87–93%; (c) arylsulfonamide, EDC·HCl, DMAP, CH_2Cl_2, room temperature, 20–95%.

By a more in-depth analysis of the data presented in Fig. 2, we could establish by a SAR study the important structural features required for the zafirlukast derivatives' activity. We performed this analysis as follows. For each of the three series of derivatives (series 1: **4a** and **4b**, series 2: **11a–12a**, and series 3: **21a–23b**, as presented in Schemes 1–3, respectively), we first compared each derivative to the parent zafirlukast and then compared amongst themselves, the compounds within the series. For series 1, the removal of the cyclopentyl carbamate group from the parent drug zafirlukast, as in compound **4b**, resulted in reduced activity (*i.e.*, reduced growth inhibition). The elimination of a methyl group from the arylsulfonamide scaffold of **4b** (compound **4a**) had no diminishing effect on the activity compared to **4b**. In the case of series 2, the removal of the cyclopentyl carbamate group, methoxy group from the benzoyl scaffold, and methyl group from the indole of zafirlukast resulted in compound **11b** with reduced activity. Exclusion of the methyl group from the arylsulfonamide of **11b** (compound **11a**) resulted in further loss of activity. Reinstating the methyl group of the indole of compound **11a** yielded derivative **12a** with substantially improved activity, which was comparable to zafirlukast and tetracycline. In the case of derivatives **21a**, **21b**, **22a**, **22b**, **23a**, and **23b** (series 3), permanent modifications were made in the substitution patterns of the benzoyl ring (position of methoxy and carboxylate groups) of zafirlukast. The replacement of the cyclopentyl carbamate group with a nitro moiety (compound **23b**) resulted in a

compound with superior activity to zafirlukast. The removal of the methyl group from the arylsulfonamide of compound **23b** (compound **23a**) had no undesirable effect on the activity compared to compound **23b**. Similarly, the elimination of the nitro group from compound **23b** (compound **22b**) was not detrimental to the activity of compound **22b**. Further exclusion of the methyl group from the arylsulfonamide of derivative **22b** (compound **22a**) also had no negative effect on the activity. Removal of the methyl group from the indole of compounds **22a** and **22b** resulted in derivatives **21a** and **21b**, respectively, with reduced activity in comparison.

In order to find whether the zafirlukast derivatives were selective against *P. gingivalis*, the best compounds **22b**, **23a**, and **23b** were then tested against other oral Gram-positive and Gram-negative bacteria, such as *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans*, *Streptococcus sanguinis*, *Veillonella parvula*, and *Fusobacterium nucleatum* (Fig. 3). Except for the minimal activity displayed against *S. sanguinis* and *F. nucleatum* (~20%), none of the synthesized zafirlukast derivatives exhibited any activity, which suggests antibacterial selectivity for *P. gingivalis*.

To determine if the antibacterial activity of the zafirlukast derivatives was bacteriostatic or bactericidal, we performed a colony forming unit (CFU) assay by exposing compounds **22b**, **23a**, and **23b** against *P. gingivalis* for a 24 h period followed by seven days of incubation (Fig. 4). *P. gingivalis* incubated with the antibiotics tetracycline (T, 2.25 μM) and zafirlukast (Z, 25 and 50 μM) were used as positive controls, whereas *P. gingivalis* incubated with DMSO was used as a negative control. We found compounds **23a** and **23b** to be bactericidal at 100 μM

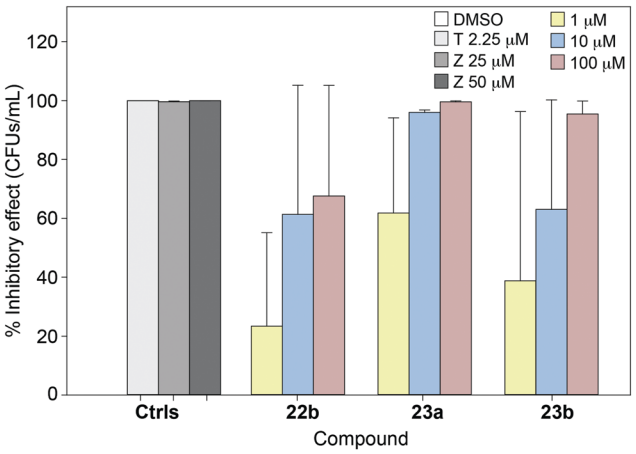


Fig. 4 Bactericidal effect of the zafirlukast derivatives against *P. gingivalis*. *P. gingivalis* (10^6 cells) exponentially growing in appropriate medium and under anaerobic conditions was exposed to zafirlukast (Z) derivatives **22b**, **23a**, and **23b** (1, 10, and 100 μM) for 24 h. Diluted bacteria [1 : 400] were seeded in blood agar plates and incubated for 7 days, and colony-forming units (CFUs) were counted. Percentage of inhibitory effect calculated comparing treatment groups *versus* bacteria only in growth medium. Bacteria incubated with antibiotics tetracycline (T, 2.25 μM) and zafirlukast (Z, 25 and 50 μM) were used as positive controls. Bacteria incubated with DMSO were used as a negative control. Data represent the mean of 5–6 replicates per condition.

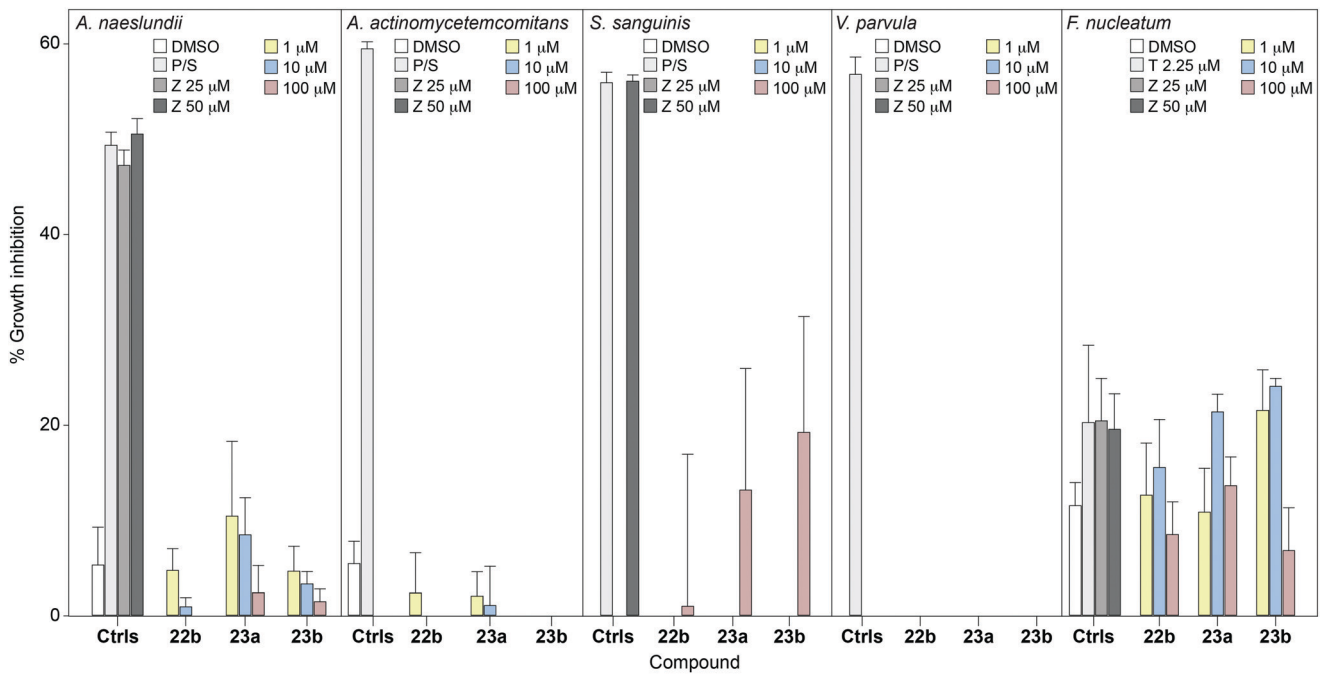


Fig. 3 Antimicrobial effect of the zafirlukast derivatives against oral bacterial species. Oral bacterial species (10^6 cells) exponentially growing in appropriate medium and under aerobic/anaerobic conditions were exposed to zafirlukast (Z) derivatives **22b**, **23a**, and **23b** (1, 10, and 100 μM) for 24 h. Bacteria incubated with an antibiotic (2.25 μM of tetracycline (T) equivalent to 1 μg mL^{-1} for *F. nucleatum* or 100 U mL^{-1} penicillin + 100 μg mL^{-1} streptomycin (P/S) for all other bacteria) or zafirlukast (Z, 25 and 50 μM) were used as positive controls and bacteria incubated with DMSO were used as a negative control. Data represent the mean inhibitory effect *versus* bacteria growing only in medium from 6 replicates per condition determined by a colorimetric WST-1 assay.

against *P. gingivalis*. With its 100% growth inhibition at 10 μM, compound **23a** displayed even superior activity to the control drug zafirlukast used at higher concentrations (25–50 μM). Compound **23a** was found to be extremely potent as it exhibited close to 60% growth inhibition even at 1 μM.

Effect of the zafirlukast derivatives on the cell viability of oral epithelial cells

The oral epithelial cells provide a number of important functions such as protection against microorganisms, external aggression, and toxic materials, and prevention against mechanical damage. The drugs designed to target bacterial cells may cause unwanted toxicity to mammalian cells. Therefore, it is crucial to consider selectivity as a parameter when developing antibacterial agents. To determine the selectivity of the zafirlukast derivatives synthesized, we tested the most active compounds **22b**, **23a**, and **23b** against immortalized oral keratinocyte cell line OKF6, along with zafirlukast as a control.[40–43] A preliminary LIVE/DEAD assay was performed after incubating the cells with compounds **22b**, **23a**, and **23b** for 24 h (Fig. 5). For compounds **22b**, **23a**, and **23b**, we observed 70–90% cell viability at 1 μM. An increase in concentration from 1 μM to 10 μM resulted in more cell death with the exception of treatment with compound **23a**. The IC_{50} values for compounds **22b**, **23a**, and **23b** were also determined in a similar assay performed in triplicate using concentrations of 0, 1, 2.5, 5, 10, 15, 20, 25, 50, and 100 μM of the molecules, and were found to be 16 μM, 54 μM, and >100 μM (estimated at ~230 μM), respectively (Fig. 6 and Table S2†). The effect of the zafirlukast derivatives on the cell viability of oral epithelial cells was further confirmed by flow cytometry analysis (Fig. 7). We observed 50–70% cell viability in the case of compounds **22b**, **23a**, and **23b** in the concentration range that exhibited antimicrobial activity (1–10 μM). Based on these assays we can conclude that the synthetic derivatives **22b**, **23a**, and **23b** displayed less cytotoxicity when compared to the parent drug zafirlukast.

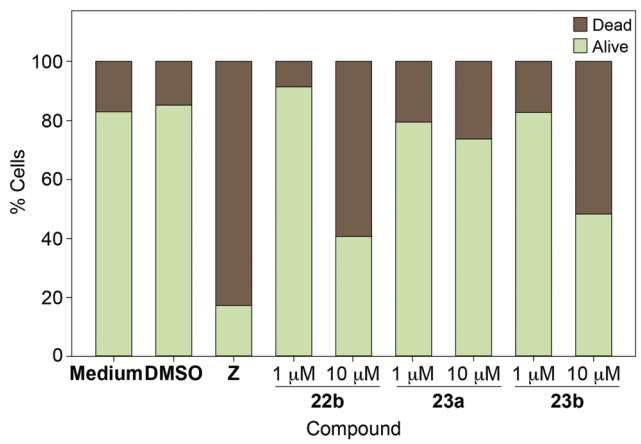


Fig. 5 Preliminary study of the effect of the zafirlukast derivatives on the cell viability of oral epithelial cells. OKF6 cells were exposed to zafirlukast (Z, 25 μM) or its derivatives **22b**, **23a**, and **23b** at 1 and 10 μM for 24 h and the cell viability was tested using trypan blue with an automated cell counter (Countess II FL. Life Technology). Trypan blue data represent the mean from two independent experiments in duplicate.

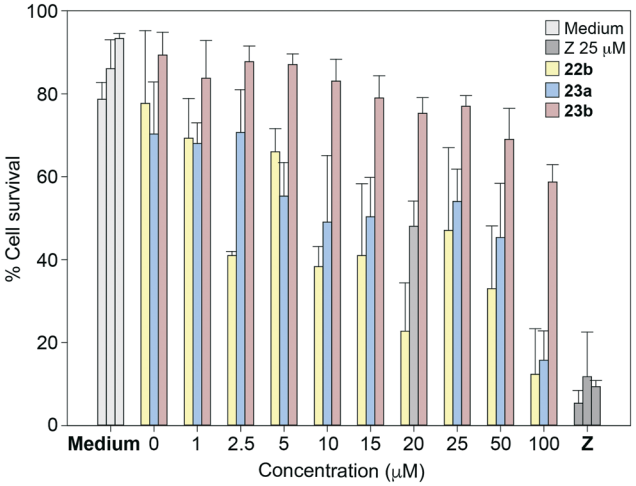


Fig. 6 Effect of the zafirlukast derivatives on the cell viability of oral epithelial cells. OKF6 cells were exposed to zafirlukast (Z, 25 μM) or its derivatives **22b**, **23a**, and **23b** at different concentrations (0, 1, 2.5, 5, 10, 15, 20, 25, 50, and 100 μM) for 24 h and the cell viability was tested using trypan blue with an automated cell counter (Countess II FL. Life Technology). Data represent the mean and SD from experiments performed in triplicate.

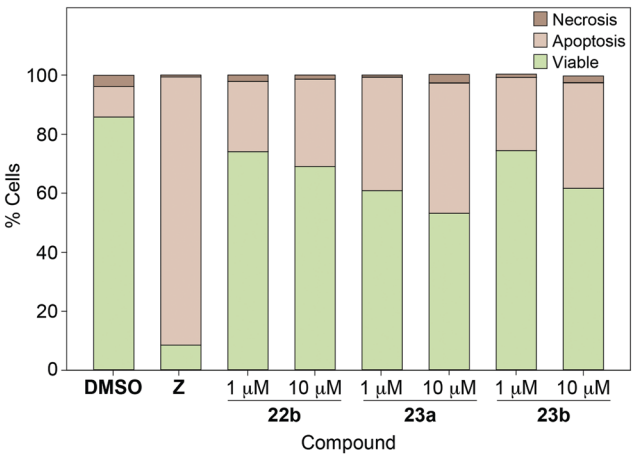


Fig. 7 Effect of the zafirlukast derivatives on the cell viability of oral epithelial cells (apoptosis and necrosis). OKF6 cells were exposed to zafirlukast (Z, 25 μM) and its derivatives **22b**, **23a**, and **23b** at different concentrations for 24 h and the cell viability was tested by flow cytometry using an FITC-Annexin V apoptosis detection kit (BD Pharmingen). The FACS data were generated by analyzing at least 10 000 events per condition in duplicate from two independent experiments.

Conclusion

In summary, a linear synthesis of novel zafirlukast derivatives with modifications in the indole, arylsulfonamide, and benzoyl scaffolds was carried out. In addition, a detailed study of the

antibacterial activity of zafirlukast derivatives **4a**, **4b**, **11a**, **11b**, **12a**, **21a**, **21b**, **22a**, **22b**, **23a**, and **23b** against *P. gingivalis* was performed. Commercially available drugs, zafirlukast (Z) and tetracycline (T), were used as positive controls. From the SAR studies, three leading candidates **22b**, **23a**, and **23b** were identified, which displayed excellent activity against *P. gingivalis* with 90–100% growth inhibition at a lower concentration of 10 μM. In most cases, these derivatives exhibited either comparable or enhanced antibacterial activity against *P. gingivalis* when compared to the control drugs zafirlukast and tetracycline. The structural modifications made in compounds **22b**, **23a**, and **23b** yielded superior activity compared to zafirlukast. From the SAR study, it was concluded that the *N*-methylindole, benzoyl ring, and arylsulfonamide scaffolds of zafirlukast were flexible to changes in their substituents and substitution patterns. The complete removal or the substitution of the cyclopentyl carbamate group as well as the elimination of the methyl group of the arylsulfonamide moiety had positive impact on the activity, whereas in the case of the benzoyl ring, we observed that changes in the substitution pattern resulted in derivatives with better activity. The best compounds were found to be selective for *P. gingivalis* as they exhibited minimal activity against other oral bacterial species. The bactericidal activity of compounds **23a** and **23b** was confirmed by exposing them to *P. gingivalis* for 24 h. In addition, these compounds also exerted minimal toxicity against oral epithelial cells. There are still some interesting SAR studies to be conducted in order to optimize the lead compounds reported in this study. The methyl group on the indole ring of the active compounds could be replaced by other alkyl chains as well as aromatic ring systems. The arylsulfonamide moiety offers opportunity to explore various *ortho*-, *meta*-, and *para*-substitution patterns with methyl groups and halogens. The nitro group on the active compounds **23a** and **23b** provides a handle for further structural modifications, as it can be reduced and tagged with a fluorescent moiety in order to investigate the mechanism of action of these molecules. Overall, the novel zafirlukast derivatives prepared in this study show promise as new and specific antibacterial agents against *P. gingivalis* and periodontal disease, and the proposed above studies are currently underway in our laboratory and additional information will be reported in due course.

Abbreviations

ATCC	American Type Culture Collection
BHI	Brain heart infusion
CFUs	Colony-forming units
LCMS	Liquid chromatography-mass spectrometry
ODs	Optical densities
SAR	Structure–activity relationship
WST-1	Water-soluble tetrazolium-1

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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