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SuFEx-enabled high-throughput medicinal chemistry

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Abstract

Optimization of small-molecule probes or drugs is a lengthy, challenging and resource-intensive process. Lack of automation and reliance on skilled medicinal chemists is cumbersome in both academic and industrial settings. Here, we demonstrate a high-throughput hit-to-lead process based on the biocompatible SuFEx click chemistry. A modest high-throughput screening hit against a bacterial cysteine protease SpeB was modified with a SuFExable iminosulfur oxydifluoride [RN=S(O)F_2] motif, rapidly diversified into 460 analogs in overnight reactions, and the products directly screened to yield drug-like inhibitors with 300-fold higher potency. We showed that the improved molecule is drug-like and biologically active in a bacteria-host coculture. Since these reactions can be performed on a picomole scale to conserve reagents, we

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Author Contributions

SK, QZ, KBS, and DWW conceived the project. SK designed the experiments. SK designed, synthesized, and characterized molecules. QZ and SK performed SOF_4 reaction. SK, AS, and JW performed the *in vitro* kinetics studies and analysis. SK, EC, and MVH performed the high-throughput synthesis and assay. SK and DWW performed the crystallography and structure analysis. AS performed the toxicity screen. ND and VN performed neutrophil assays. SK and MK performed LC-CAD analysis. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

Additional texts, figures, and tables are provided.

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anticipate our methodology can accelerate the development of robust biological probes and drug candidates.

The introduction of high-throughput screening (HTS) robotics, liquid handler systems, and assay miniaturization have revolutionized screening of bioactive molecules. Relatively inexpensive HTS processes are now routinely used in cell-based and *in vitro* assays against biomedically relevant targets. Nevertheless, compound optimization is typically necessary to improve target specificity, potency, and stability. Lead optimization relies heavily on medicinal chemists, and extensive time and labor costs remain significant hurdles for probe and drug development.

Click chemistry has found broad applications in materials chemistry, chemical biology, and drug development since the concept was first introduced in 1999[1–2]. The sulfur(VI) uoride exchange (SuFEx) represents the most recent set of ideal click chemistry transformations[3]. Specifically, aryl fluoflrosulfates ($ArOSO_2F$) and iminosulfur oxydifluorides ($RN=S(O)F_2$) are readily synthesized using two connective oxyfluoride gases, sulfuryl fluoride (SO_2F_2) and thionyl tetrafluoride ($O=SF_4$), respectively[4]. These two S^{VI}–F motifs have been successfully used as connective linkers in polymer synthesis and for construction of various functional molecules[5–7]. Sulfonyl fluoride (RSO_2F) and aryl fluorosulfate moieties have been successfully introduced into bioactive molecules in chemical biology and drug discovery[8–11], especially as covalently binding warheads[12]. However, the potential of SuFEx to unite diverse modules using an $O=SF_4$ hub has not been explored in medicinal chemistry. While the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction has been used in proof-of-concept studies on lead optimization, including the direct evaluation of biological potency[13–18], there are only a couple of drugs that contain the 1,2,3-triazole linkage, supposedly because of several drawbacks of the reaction (Figure 1). Unlike CuAAC, the sulfur(VI)-containing motifs resulting from SuFEx reactions are common in drugs; for example, more than 150 sulfonamide drugs are available on the market[19]. Here, we present a rapid and high-throughput hit-to-lead optimization process based on iminosulfur oxydifluoride SuFEx click chemistry that can be performed on picomole scale.

We focused on the SuFEx reaction between iminosulfur oxydifluoride ($RN=S(O)F_2$, *isodifluor*) and primary or secondary amines to construct a focused library of lead compound analogs (Figure 1). This series of robust and near perfect reactions was recently described for bioconjugation and DNA-encoded library construction[20], and we posited that the biocompatible reaction conditions would enable us to measure the potency of products directly using *in vitro* enzyme assays to prioritize the molecules. Additionally, the rapid and diverse analog synthesis from the most available starting materials (*i.e.*, primary and secondary amines) and the non-planer 3-dimensional structures, multiple hydrogen-bond donors/acceptors, drug-like lipophilicity, and stability in biological conditions of the products are ideal for medicinal chemistry (Figure 1).

Our proof-of-concept started with a modest inhibitor (cmpd **1**, IC_{50} = 14 μM) of the cysteine protease SpeB, a virulence factor secreted from the bacterial pathogen *Streptococcus pyogenes*, previously identified in our HTS campaign (Figure 2a)[21–22]. Although peptidic SpeB inhibitors were reported, such as E64[23–24], potent small molecule inhibitors have not

been developed against SpeB. In preliminary SAR studies, introduction of an (*S*)-benzyl moiety (cmpd **2**, Figure 2a) improved the potency to 2.1 μM. The SpeB:**1** co-complex x-ray structure[22] and initial SAR campaign (Table S1) suggested that additional surface pockets on SpeB were accessible for compound optimization via extension of **2** from the meta positions of both benzyl rings. An isodifluor diversification handle was therefore introduced at the meta position of either benzyl moieties of **2** to generate **3** and **4** (Figure 2b). These molecules with an isodifluor hub were subsequently reacted with a panel of 230 amines to generate 460 analogs overnight using DMSO:PBS = 1:1 as a solvent and incubate at 37 °C (Figure 2c). The representative reactions monitored using LC-CAD-MS^2 are shown in the Supporting Data and Table S3. It should be noted that the reactions between isodifluor-containing molecules with the amines showed an improved yield when PBS (pH 7.4) was added to the solvent (Table S2).

The reaction products were directly screened for SpeB inhibition with an established kinetic fluorogenic substrate assay[22–23]. Scatter plots of the screening results are shown in Figure 2c. All 460 amine structures and the corresponding SpeB inhibition are provided in Tables S4 & S5. Additionally, the panel of amines alone (absence of **3** or **4** in reaction), the reaction condition, and the fluoride ion by-product (Figure 1) were assessed for inhibition of SpeB hydrolysis, with no appreciable effect on proteolysis or the assay observed (Figures S1 & S2). Molecules selected based on potency, lipophilicity, and molecular weight were manually re-synthesized and purified on milligram scale. We observed a correlation between potency estimated in the initial screen and those of re-synthesized compounds (Figure S3). Structures of representative molecules with improved IC_{50} values are shown in Figure 2d.

With significantly improved SpeB inhibitors in hand, we next assessed if miniaturization of the SuFEx reaction was feasible using an Echo Acoustic liquid handler. A strong correlation in inhibitory potency was observed between the picomole-scale (1536-well, 2 μL, 200 μM of isodifluor compound, 400 pmol) and nanomole-scale (96-well, 50 μL, 10 nmol) syntheses, demonstrating the successful miniaturization of the library construction (Figures 2e & S4). Importantly, unlike previously reported nanoscale medicinal chemistry attempts[25], our sub-nanoscale SuFEx-based library synthesis does not require specialized equipment, such as dry-boxed liquid handlers and highly sensitive mass spectrometry for the biological assay. Our SuFEx-based format can be readily adapted in screening facilities with standard HTS robotics and liquid handler systems.

We next characterized an improved compound **5** with biochemical and biophysical methods to substantiate the improved potency. Enzyme kinetics showed that **5** is a reversible, competitive inhibitor with $K_i = 18 \pm 1$ nM (Figures S5 & S6). The improved binding affinity was further validated by surface plasmon resonance and differential scanning fluorometry (Figures S7 & S8). We determined the x-ray crystal structure of SpeB in complex with **5** to elucidate the origin of improved inhibition (Figures 3 & S9, Table S6). Interestingly, **5** binds SpeB in a U-shaped conformation with an intramolecular CH-**π** interaction[26–27] between the benzyl moiety and a hydrogen on the piperidyl group that likely contributes the binding confirmation (Figure S10). Compound **5** binds within the SpeB active site whereby the carbonyl oxygen is oriented toward the SpeB oxyanion hole created by the main-chain nitrogen atoms of residues Cys192 and Val193 (Figure 3).

Based on biological stability and solubility in PBS (Table S8), compound **7** was selected for further biological characterization. As shown in Figure 4a, **7** is stable against human liver microsomes *in vitro*, soluble in PBS, selective for SpeB (over other cysteine proteases), non-cytotoxic, and adheres to Lipinski's rules. We tested the effect of inhibitor **7** in an established neutrophil killing assay, wherein SpeB activity provides relative resistance to *S. pyogenes* against human neutrophils[28–29]. Wild-type (WT) *S. pyogenes* (M1 serotype strain 5448) and a corresponding isogenic mutant strain lacking SpeB (ΔSpeB) were preincubated with **7** prior to introduction of freshly isolated neutrophils from human blood. The presence of **7** decreased the viability of WT *S. pyogenes* in a concentration-dependent manner, while no similar drug effect of **7** occurred in the ΔSpeB mutant strain (Figure 4b).

In conclusion, we provide a proof-of-concept of high-throughput process to improve potency of an HTS hit molecule to generate a drug-like, biologically active molecule using biocompatible SuFEx click chemistry. This study highlights the utility of SuFEx chemistry for rapidly generating diversified molecules for hit-to-lead applications and shows the potential of the combination of click chemistry, miniaturized synthesis, and direct evaluation of biological potency. Efforts to improve and expand the method are underway to develop an HT medicinal chemistry platform applicable for routine use[30]. Molecules described here represent the first potent and selective small molecule SpeB inhibitors and can be used to address biological functions of this protease in cellular and animal models and establish as a potential target for the development of treatments to combat streptococcal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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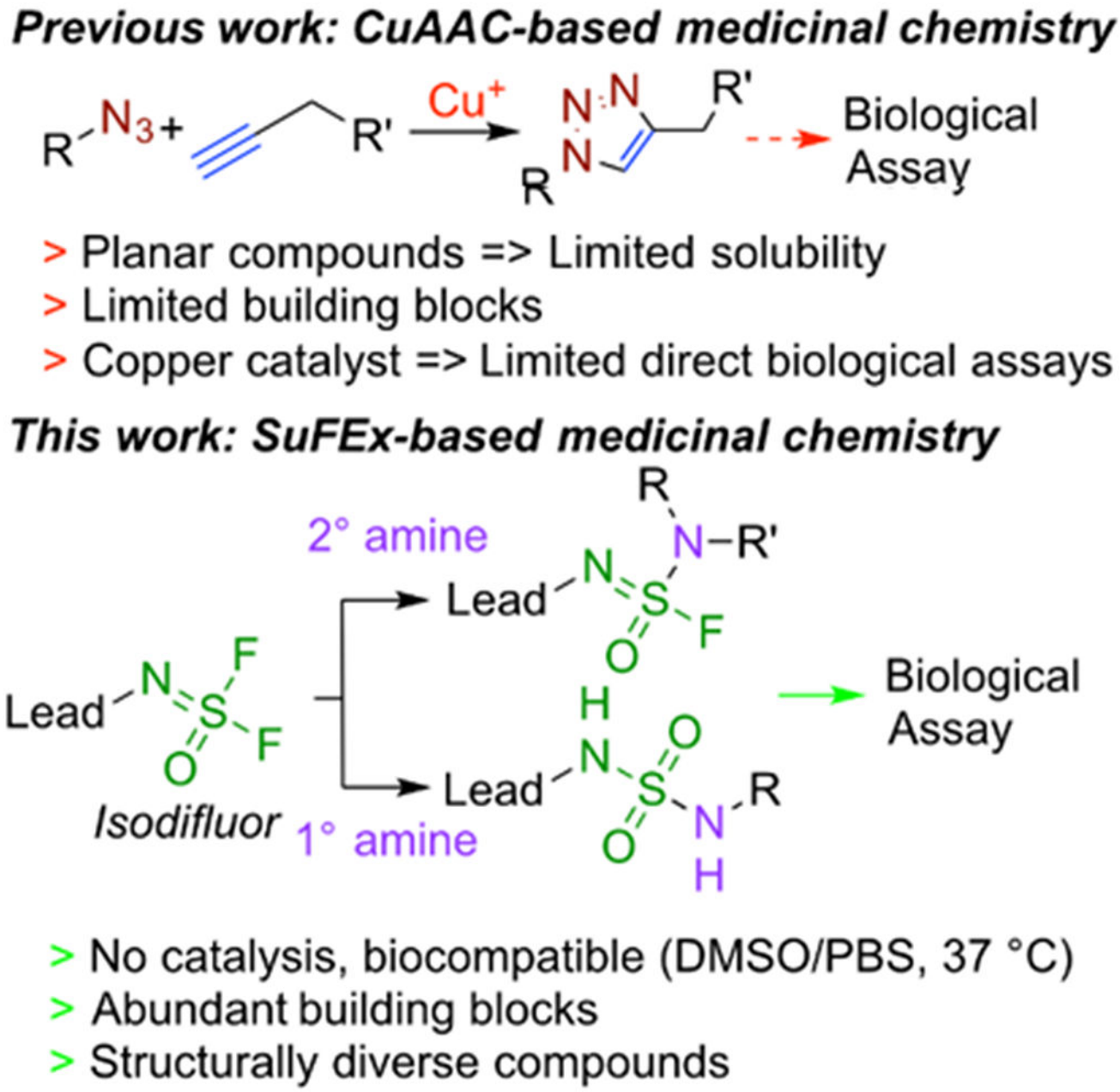

Figure 1.

Comparison of CuAAC- and SuFEx-based medicinal chemistry campaigns. Lead molecules can be modified with an iminosulfur oxydifluoride (*isodifluor*) motif and reacted with a collection of primary and secondary amines to generate a diversified library.

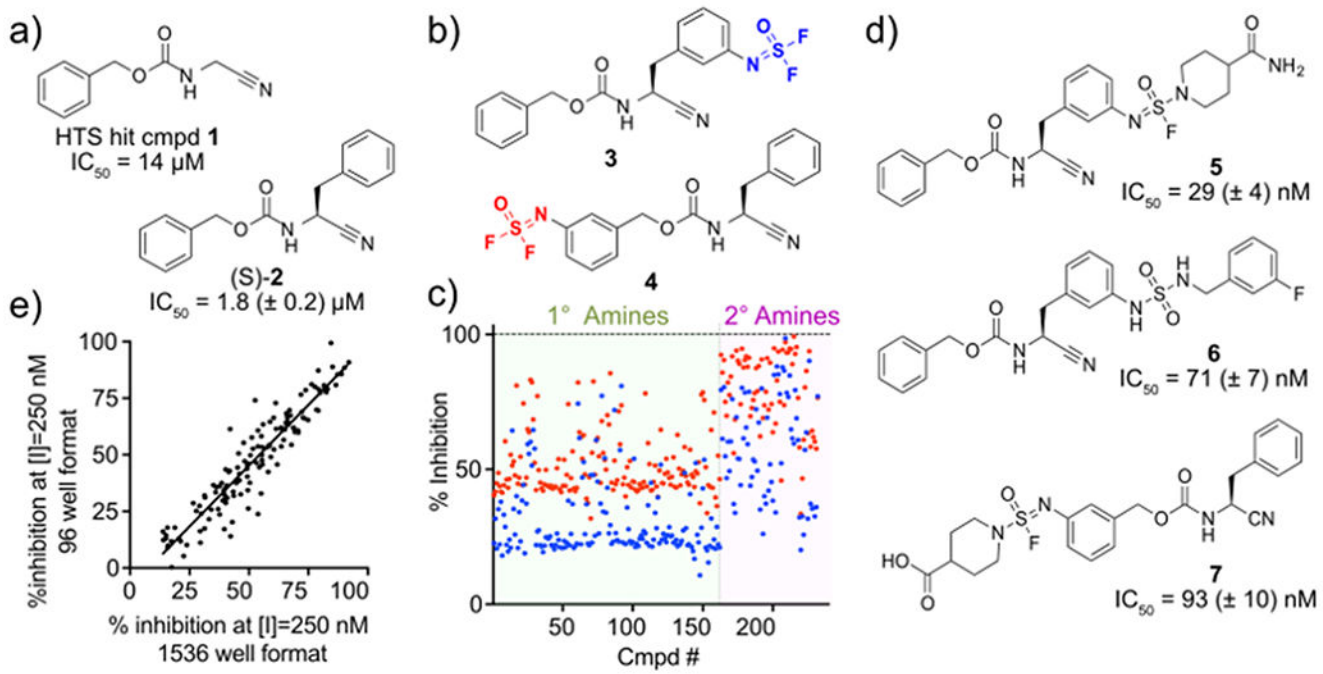

Figure 2.

Focused library construction and screening. (a) Structures of HTS hit SpeB inhibitor **1** and its analog (*S*)-**2**. (b) Molecules with isodifluor diversification handle, and (c) scatter plot. Inhibition % at 250 nM (compound **3,** ●) or 2 μM (compound **4,** ●) are plotted. (d) Representative improved inhibitors and SpeB inhibition potency. (e) Correlation between picomole-scale and nanomole-scale syntheses.

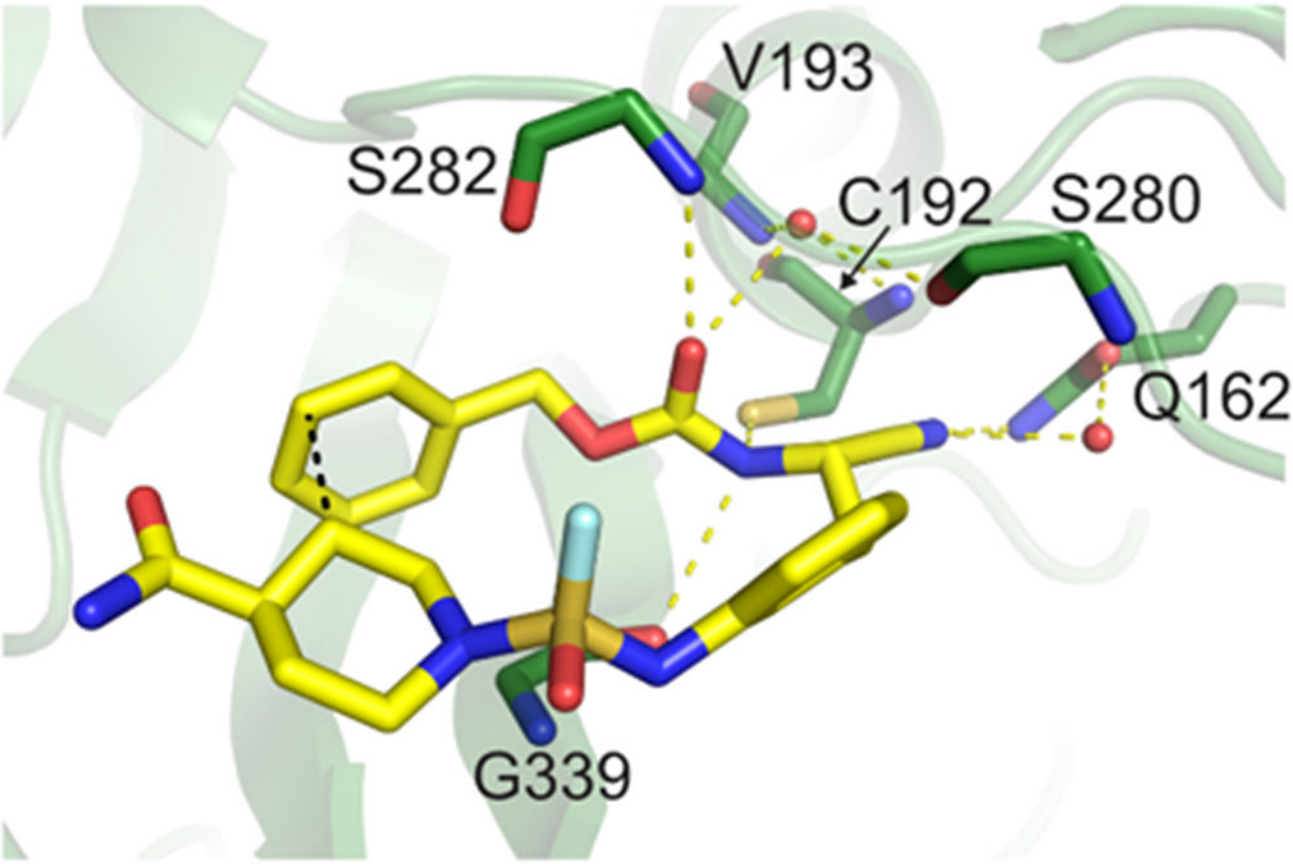

Figure 3.

X-ray structure of SpeB-compound **5** structure (PDB ID 6UQD). Cmpd **5** (yellow carbon) bound to the SpeB (green carbon) are shown as sticks (red oxygen, blue nitrogen, mustard sulfur, teal fluorine).

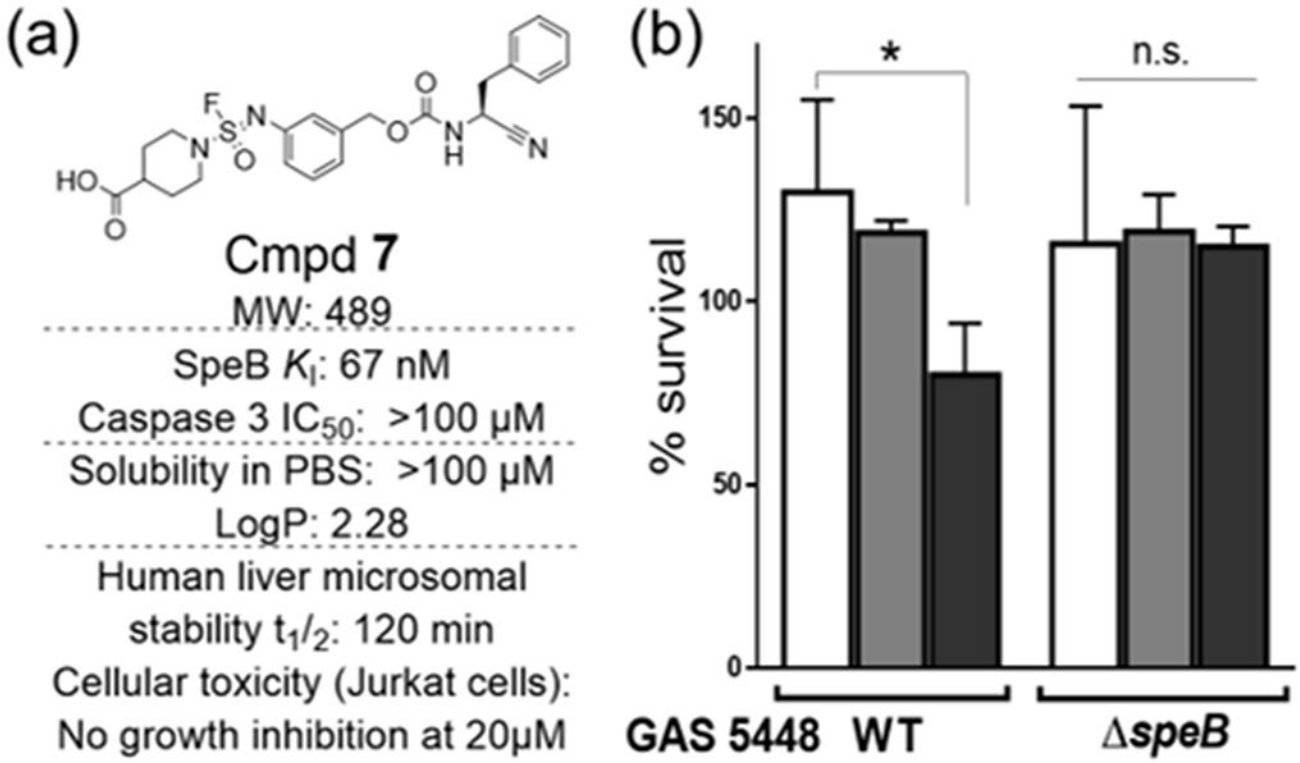

Figure 4.

Improved SpeB inhibitor **7** is drug-like and biologically active in bacteria-neutrophil co-culture. (a) Drug-likeness of cmpd **7**. Solubility in PBS[31–32], caspase activity[33], microsomal stability and cellular toxicity were measured as described[34], LogP was predicted with ChemDraw Ultra 17.1. (b) cmpd **7** prevents *S. pyogenes* WT GAS5448 from neutrophil killing by the inhibition of SpeB; however, no effect is observed on the ΔSpeB strain. Vehicle (white bar), 7 (20 μM (light gray bar), or 40 μM (dark gray bar)). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test, *≤ 0.05.