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Dual Dehydrosqualene/Squalene Synthase Inhibitors: Leads for Innate Immune System Based Therapeutics

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With the rapid rise in bacterial drug-resistance,^[1] there is great interest in developing innovative approaches to anti-infective therapy. For example, in the United States, more people die from *Staphylococcus aureus* infections than die from HIV/AIDS.^[2] Alternative approaches are thus of interest, and as discussed in a recent NRC Report,^[1b] these include targeting virulence factors, as well as boosting innate immunity. In recent work^[3] we showed that blocking formation of the carotenoid virulence factor staphyloxanthin with BPH-652 (**1**, Scheme 1) rendered staph bacteria non-infective and susceptible to immune system clearance mediated by reactive oxygen species, and in other work,^[4] we showed that inhibiting squalene biosynthesis in neutrophils with **2** generated anti-bacterial, neutrophil extra-cellular traps or NETs. The targets involved, dehydrosqualene synthase (CrtM, for **1**) and squalene synthase (SQS, for **2**), are both involved in the first committed steps in carotenoid and sterol biosynthesis, the conversion of two farnesyl diphosphate molecules to form presqualene diphosphate, Figure 1 a. These findings led us to contemplate that it might be possible to develop dual-activity CrtM/SQS inhibitors that simultaneously block formation of the *S. aureus* virulence factor staphyloxanthin - removing the golden protective shell (Figure 1 b) of the bacterium, while at the same time stimulating host anti-microbial NET formation (Figure 1 c): a novel, dual-targeting approach to immunotherapy. Here, we report the discovery of such leads.

We first carried out an *in silico* high-throughput search for new CrtM inhibitor leads, since current inhibitors such as **1** are poor NET inducers (Table 1). Likewise, the use of statins to block pigment formation is not feasible since the K_i for inhibition of *S. aureus* HMG-CoA reductase by statins is $\sim 10^4$ larger than for inhibition of host HMG-CoA reductase, due to large class I/class II enzyme structural differences.^[5] We used the CrtM:1 structure reported

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previously^[3] (PDB ID code 2ZCQ) minus the phosphonosulfonate ligand, retaining 2 Mg²⁺ and water molecules within 5 Å around **1**. We used the Virtual Screening Workflow in Glide,^[6] and screened 2,372 compounds from the Life Chemical antibacterial library (<http://www.lifechemicals.com>) and 14,400 compounds from the Maybridge HitFinder library (<http://www.maybridge.com>). Hits **3-5** from the first library and **6-8** from the second library are shown in Scheme 1, together with their Glide scores and K_i values. Each of these compounds has a carboxylic acid attached either to a benzene ring or other hydrophobic group, plus, in each case there is a more distal, aromatic feature. We attempted crystallization of **3-8** with CrtM and obtained crystals that diffracted well for **6** (2.07 Å) and **8** (2.00 Å). Full crystallographic data and structure refinement details are given in Table S1 and Methods in the Supporting Information. Ligand electron densities are in the Supporting Information Figure S1 a, b.

We show in Figure 2 a, b the structures of **6** and in Figure 2 c, d the structures of **8**, bound to CrtM. In both cases, what can immediately be seen is that the more distal, hydrophobic, diphenylmethane (**6**) or diphenylsulfide (**8**) side-chains bind to both the S1 as well as the S2 hydrophobic pockets^[7] in CrtM that are involved in FPP substrate binding. Furthermore, in both cases, one phenyl group is centered at ~C10 in the S2 FSPP (S-*thiolo*-farnesyl diphosphate, an FPP-like inhibitor) side-chain position, while the second phenyl group overlaps the position occupied by the chain terminus of FSPP, in S1. However, with **6**, the carboxyl group has electrostatic interactions with R171 and R265 in the polar group region in S2, Figure 2 b while in **8**, interactions are with the carboxyl and Mg_A²⁺ in S1, Figure 2 d. Both inhibitors have good activity against CrtM (~300 nM) and > 20 μM IC₅₀ values against three human cell lines,^[3] Table 1. However, these initial screening hits were not active in an *S. aureus* pigment inhibition assay (IC₅₀ > 100 μM), Table 1.

Based on the preponderance of *ortho*-substituted benzoic acids in the HTS hits (3/4 compounds having, on average, a CrtM K_i ~ 400 nM, we next carried out a similarity search in PubChem^[8] (based on **3-8**) to find other leads. An interesting result was that the compound S-*thiolo*-farnesyl-benzoic acid, Salirasib (**9**), was returned as a similar compound. Salirasib blocks Ras signaling^[9] and is currently in clinical trials for various cancers, and since it is orally available, appeared an interesting potential lead. Salirasib (**9**) had a K_i = 110 nM in the CrtM enzyme assay, Table 1, but *S. aureus* pigment inhibition was again poor, IC₅₀ = 75 μM. We reasoned that a longer chain (geranylgeranyl) analog, **10**, might have better enzyme inhibition activity, since it is known that geranylgeranyl diphosphate can bind to the S2 site,^[7] and indeed, **10** had a 28 nM K_i versus CrtM, as well as a 4 μM IC₅₀ in pigment inhibition. We then produced five analogs, **11-15**, based on **10**. Among them, **14** had a 120 nM K_i in CrtM inhibition and an IC₅₀ of 120 nM in pigment inhibition. We attempted to crystallize each of **9-15** bound to CrtM using both soaking as well as co-crystallization, but found no complete ligand densities. Soaking **14** yielded crystals that diffracted to 2 Å, but there was missing density in the benzoate region, due to structural disorder. We thus made the Br-analog, **16**, and one crystal diffracted to 3.00 Å, Table S1. Although this is only moderate resolution, the electron density (Supporting Information Figure S1 c) clearly indicates that the long alkyl chain binds to the S2 site, Figure 2 e (in orange) and is essentially superimposed on the S2 FSPP side-chain (in green, Figure 2 e), while the benzyl group forms a T-shaped π-stacking interaction with Y41 and the bromobenzoate fragment binds in the S1 site, Figure 2 f, interacting with Mg_B²⁺.

We tested **9-16** for activity in human cell growth inhibition in order to see whether or not these compounds were highly toxic. We used a “therapeutic index” (TI) defined as: TI (therapeutic index) = IC₅₀ (pigment)/IC₅₀ (human cell), finding that (Table 1) the most potent leads (**13**, **14**) also had the best TI (~ 100). We then screened the small library of compounds synthesized for activity in SQS inhibition. Results are shown in Table 1. The

most active compound that also blocked pigment formation and had a good TI was **14**, which had a $K_i \sim 200$ nM in SQS inhibition. More importantly, 5 μ M **14** caused extensive NET formation with phorbol 12-myristate 13-acetate (PMA)^[4] stimulated neutrophils, Figure 2 g, h, as evidenced by staining with monoclonal antibodies against neutrophil myeloperoxidase (MPO).^[4] The potent CrtM/SQS inhibitor **16** was also active in NET formation (Table 1), but unlike **14** also inhibited *S. aureus* growth. This is similar to the observation that other *meta*-substituted benzoic acids inhibit *S. aureus* growth,^[10] suggesting that direct antibacterial activity, virulence factor inhibition as well as innate immune system activation via NET formation may all be targeted by a single compound. Taken together, these results suggest new avenues for treatment of *S. aureus* infections in the current era of increasing resistance to conventional antibiotic therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Head-to-head prenyltransferase drug targets. a) Schematic illustration of the reactions catalyzed by two head-to-head prenyl transferases: CrtM and SQS. The end products are the carotenoid, staphyloxanthin or the sterol, cholesterol. b) Staphyloxanthin gives *S. aureus* its (eponymous) gold coloration, and is a major virulence factor. c) Blocking cholesterol biosynthesis in neutrophils at the level of squalene synthase results in formation of NETs, neutrophil extra-cellular traps, that have potent anti-bacterial activity.

Figure 2.

Crystal structures and NETs formation. a) Superimposition of **6** (cyan, PDB ID code 3TFN) on two FSPP molecules (green and yellow; PDB ID code 2ZCP). b) Polar CrtM residues interacting with **6**. c) Superimposition of **8** (pink, PDB ID code 3TFP) with FSPP (PDB ID code 2ZCP). d) Mg_A^{2+} and CrtM residues interacting with **8**. e) Superimposition of **16** (orange, PDB ID code 3TFV) with FSPP (PDB ID code 2ZCP). f) Mg_B^{2+} and CrtM residues interacting with **16**. g) PBS control with phorbol myristate stimulated neutrophils. h) Extensive sheets of NETs are formed on activation of neutrophils with 5 μM **14**.

Scheme 1.
Structures of **1** to **16**.

Table 1

Enzyme, pigment, cell growth inhibition and NET induction results.

Compounds	hSQS Ki (μ M)	CrtM Ki (μ M)	Pigment inhibition (μ M) ^[a]	Cancer average (μ M) ^[b]	NET formation ^[c]	TI ^[d]
1	0.37	0.09	0.12	>200	-	~0
4	0.78	0.18	> 100	32	ND	< 0.3
6	0.53	0.27	> 100	15.7	ND	< 0.2
8	9	0.34	100	101	ND	< 1
9 (salirasib)	0.37	0.11	75	15.8	+	0.2
10	0.3	0.028	4	15.6	+	3.9
11	0.06	0.052	0.92	22	-	24
12	2	0.028	9.0	29	-	39
13	1.25	0.16	0.15	13.3	+	89
14	0.2	0.12	0.12	16	+++	133
15	0.3	0.09	ND	10.4	+	ND
16	0.2	0.08	ND	37	++	ND

^[a] ND indicates not determined since there was bacterial cell growth inhibition, so pigment inhibition could not be accurately measured.

^[b] Average for MCF-7, NCI-H460 and SF-268 cell lines as described in ref. [3].

^[c] NET formation was not determined if there was no pigment inhibition.

^[d] TI was not determined if there was bacterial growth inhibition.