Inhibitor of streptokinase gene expression improves survival after group A streptococcus infection in mice

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The widespread occurrence of antibiotic resistance among human pathogens is a major public health problem. Conventional antibiotics typically target bacterial killing or growth inhibition, resulting in strong selection for the development of antibiotic resistance. Alternative therapeutic approaches targeting microbial pathogenicity without inhibiting growth might minimize selection for resistant organisms. Compounds inhibiting gene expression of streptokinase (SK), a critical group A streptococcal (GAS) virulence factor, were identified through a high-throughput, growth-based screen on a library of 55,000 small molecules. The lead compound [Center for Chemical Genomics 2979 (CCG-2979)] and an analog (CCG-102487) were confirmed to also inhibit the production of active SK protein. Microarray analysis of GAS grown in the presence of CCG-102487 showed down-regulation of a number of important virulence factors in addition to SK, suggesting disruption of a general virulence gene regulatory network. CCG-2979 and CCG-102487 both enhanced granulocyte phagocytosis and killing of GAS in an in vitro assay, and CCG-2979 also protected mice from GAS-induced mortality in vivo. These data suggest that the class of compounds represented by CCG-2979 may be of therapeutic value for the treatment of GAS and potentially other Gram-positive infections in humans.

fibrinolysis | plasminogen | *Streptococcus pyogenes*

Current antibiotics generally act by interfering with critical biological processes in the pathogen, resulting in death or growth arrest. These mechanisms exert strong selective pressure that favors emergence of antibiotic-resistant strains (1). An alternative strategy is to suppress pathogen virulence without inhibiting growth, thereby attenuating the strong selection for resistance exerted by traditional bacteriostatic or bactericidal antibiotics. This approach has been applied to cholera, identifying a small molecule that blocks the production of two critical virulence factors, cholera toxin and the toxin coregulated pilus, by inhibiting the expression of their transcriptional regulator ToxT. This compound was then shown to protect infant mice from intestinal colonization by *Vibrio cholerae* (2).

Streptococcus pyogenes or group A *Streptococcus* (GAS) is an important human pathogen that is estimated to cause ~700 million symptomatic infections per year worldwide (3). The clinical spectrum includes both mild conditions, such as pharyngitis, scarlet fever, and impetigo, as well as life-threatening disease, such as toxic shock-like syndrome and necrotizing fasciitis (4, 5). Streptococci are a diverse group of Gram-positive bacteria infecting humans and various other animals (6). *S. pyogenes* is highly specific to its human host, presumably because of the activity of species-specific virulence factors, including streptokinase (SK) (7). SK binds to the inactive zymogen plasminogen, resulting in the production of active plasmin, the central protease of the fibrinolytic system, through a coupling of conformational and proteolytic activation (8, 9).

The interaction of SK with plasminogen is highly species-specific, with the SK expressed by human GAS isolates active only against human plasminogen (10–13). Mice expressing human plasminogen exhibit markedly increased mortality after GAS infection, which is largely abrogated by deletion of the SK gene (*ska*) (7). These results and others show that SK is a key GAS virulence factor (14). We now report the identification of small-molecule inhibitors of GAS SK expression by high-throughput chemical screening and characterize the efficacy of these compounds both in in vitro and in vivo murine infection models.

Results

SK Expression Inhibitors Identified by High-Throughput Screening.

We previously reported that the species-specific interaction of SK with human plasminogen is critical to the pathogenicity of GAS in an in vivo murine infection model (7). These results suggested that inhibition of *ska* gene expression might provide an effective strategy for the treatment of GAS infection. To identify small molecules as candidates for this approach, a high-throughput screening assay was developed based on a kanamycin resistance gene under control of the *ska* promoter (strain SKKanGAS). Compounds were tested for the ability to inhibit SKKanGAS growth in the presence of kanamycin. A duplicate screen using the constitutively kanamycin-resistant strain UMAA2641 (15) served as control to detect compounds exhibiting nonspecific inhibition of GAS growth.

A total of 55,000 compounds at concentrations ranging from 5 to 10 μ M were screened, leading to the identification of 95 compounds that exhibited $\geq 50\%$ SKKanGAS inhibition of growth in the presence of kanamycin (i.e., inhibited SK promoter activity), with $<10\%$ inhibition of the UMAA2641 control strain. Dose response and IC_{50} analysis for these 95 compounds identified 20 with pIC_{50} values [$-\log(IC_{50})$] of >4.5 for SKKanGAS growth inhibition in the presence of kanamycin and IC_{50} values for control growth inhibition that are at least 10-fold higher.

The 20 compounds were then prioritized for retesting based on lipophilicity [calculated $\log P$ (ClogP) < 6] and molecular mass (<500 Da). Compounds with these physical properties have the greatest likelihood of being bioavailable based on Lipinski's Rule of 5 (16), and they make better starting points for medicinal chemistry optimization, which often requires adding molecular mass and lipophilicity (17). Among those compounds passing these criteria, eight were available for purchase from commercial suppliers (Fig. 1, structures) and retested as fresh powders. Among this cohort, compounds A, G, H, and J all inhibited

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Fig. 2. Effect of CCG-2979 and CCG-102487 on SK expression and GAS growth. (A) Effects of CCG-2979 and CCG-102487 on the production of SK activity. Normalized SK activity of GAS treated with CCG-2979 or CCG-102487 at concentrations from 5 to 100 μ M (SK activity of culture media was divided by OD₆₀₀ and then normalized to the value for DMSO-treated GAS, which was defined as 100%). The value was presented as mean $\pm$ SE for a total of 18 samples (pooled from six independent experiments with triplicates). (B) GAS growth as measured by OD₆₀₀ of an overnight culture in the presence of CCG-2979 at concentrations of 5–100 μ M normalized to the OD₆₀₀ of control GAS grown in DMSO vehicle alone. The value was presented as mean $\pm$ SE for a total of 18 samples (pooled from six independent experiments with triplicates). (C) Growth curves for UMAA2616 in the presence of 0.1% DMSO, 50 μ M CCG-2979, or 50 μ M CCG-102487. Mean and SE for a total of six samples (two independent experiments with triplicates) are presented for each time point. (D) Decreased GAS resistance to phagocytosis in the presence of CCG-2979 or CCG-102487. Each point represents an individual experiment.

scriptional regulator and hypothetical membrane protein in addition to the putative CAAX family protease. The second operon, *Spy1714-1717*, encodes elements of a putative copper exporting system, and the third operon, *Spy2172-2174*, encodes a regulator and two putative membrane-associated proteins (Fig. 3C and Table S1).

CCG-2979 Protects Mice from GAS Infection Mortality. In vivo efficacy was evaluated in a model of GAS infection in which expression of a human plasminogen transgene renders mice markedly susceptible to GAS infection, presumably because of

the species-specific interaction between SK and human plasminogen (7). Human PLG *Tg*⁺ mice were inoculated s.c. with UMAA2616 and then treated by i.p. delivery with daily doses of CCG-2979 (5 or 40 μ g) starting 24 h after infection. Improved survival was observed for both the 5- ($P < 0.038$) (Fig. 4A) and 40- μ g ($P < 0.011$) (Fig. 4B) doses of CCG-2979 ($P < 0.002$ for the pooled data) (Fig. 4C). Although a statistically significant improvement in survival was not observed with compound CCG-102487, analysis of the pooled data from all experiments by a pairwise multiple comparison procedure to correct for multiple observations (Bonferroni method) showed statistically significant

Fig. 3. The effect of CCG-102487 on the GAS gene expression program. Growth curves for MGAS2221 in the presence of CCG-102487 (46.7 μ M) or DMSO vehicle alone as determined by OD (A) or cfu (B). (C) Graphic summary of gene expression changes. Genes down-regulated on treatment with CCG-102487 are indicated in green, and genes up-regulated on treatment with CCG-102487 are indicated in red. Genes of known homology or function were included. Complete data for gene expression changes are summarized in Table S1.

Table 1. Summary of expression microarray data: total transcripts detected by the microarray analysis and altered by CCG-102487 treatment

	Number of transcripts
All nonredundant probes on the chip	1,872
Transcripts detected during at least one experimental condition	1,702
Transcript significantly changed during at least one time point as a result of treatment with CCG-102487 (ratios above or below 1.5 and P values ≤ 0.05)	536
Changed transcript excluding phage genes	490

protection for compound CCG-2979 treatment vs. control ($P < 0.004$) (Fig. 4D).

Discussion

Antibiotic resistance is an increasing problem for the treatment of most human pathogens. Although GAS resistance to penicillin has not yet been reported, macrolide resistance is increasing worldwide, with frequencies as high as 48% in some populations (21–23). Using high-throughput molecular screening, we identified a class of compounds that inhibits *ska* expression without perturbing bacterial growth, an approach that might be less likely to select for antibiotic resistance.

Of note, we also showed reduced mortality in mice treated with one of these compounds (CCG-2979) in an in vivo infection model. It was significant that we could observe protective effect of CCG-2979 even without extensive effort to optimize the dosing and delivery regimen, underscoring the great potential of this chemical series of compounds in treating GAS infection. CCG-102487 failed to show in vivo efficacy, although it showed inhibitory effects on expression of a number of virulence genes and decreased GAS resistance to phagocytosis. The different in vivo efficacies of the two compounds are likely caused by differences in pharmacokinetic properties rather than intrinsic potency. Analysis of GAS exposed to an analog of CCG-2979 (CCG-102487) identified expression changes in a number of genes, including SK and other known virulence factors, suggesting that these compounds may interact with a gene regulator (s) that controls gene expression for a broad spectrum of genes.

Although CCG-2979 and CCG-102487 exhibit their effects at relatively high concentrations (30–50 μ M), these compounds have not been optimized for potency. CCG-2979 is a screening hit, and CCG-102487 is one of the first commercially available analogs selected. It is clear that this chemical series will require substantial medicinal chemistry optimization to maximize potency at inhibiting SK expression without introducing bacterial growth suppression or toxicity in mammalian cells. Nonetheless, the in vivo activity observed with CCG-2979 without obvious toxicity suggests that development of such improved compounds should be feasible.

Although reduction of SK expression alone could account for the observed in vivo protection from GAS-induced mortality (7), alterations in the expression of other genes by this class of

compounds might also contribute. One of the genes down-regulated by CCG-102487, *fasA*, belongs to the two-component system fibronectin/fibrinogen binding/hemolytic activity/streptokinase regulator (*FasBCAX*). *FasBCAX* and control of virulence (*CovRS*) genes are major two-component systems that are both known to regulate *ska* expression (24–26). *CovRS* inhibits (24), whereas *FasBCAX* induces *ska* expression (25). Other targets in addition to *ska* were also down-regulated by CCG-102487, including streptolysin S and fibronectin-binding proteins (25), suggesting that some or all of these changes could be secondary to the inhibition of *FasBCAX*. Streptolysin S is a powerful hemolysin that can impede host neutrophil recruitment by inhibiting the production of chemotactic signals (27).

Other changes in gene expression induced by CCG-102487 that could contribute to the protective effect of this class of compounds in vivo include alterations in genes central to carbohydrate use (e.g., *amyA*, *amyB*, *malC*, *malD*, *malE*, *malM*, and *malX*) (28–31) and genes regulating nucleoside transport and metabolism (e.g., *purN*, *purD*, *pyrF*, and *pyrE*) (32, 33). The inhibition of GAS resistance to phagocytosis by CCG-2979 and CCG-102487 could be attributed to the observed down-regulation of one or more virulence factors known to be important for this process, including Sda1, SIC, M protein, or C5a peptidase (*scpA*) (20, 34–38). SpeB, a cysteine protease that has been reported to cleave and inactivate Sda1, was modestly up-regulated by CCG-102487, which would be predicted to decrease the ability of GAS to spread systemically (37, 39).

Taken together, these data suggest that the class of compounds represented by CCG-2979 and CCG-102487 target a network of virulence factors including SK, resulting in significant attenuation of GAS pathogenicity and accounting for the protection from GAS-induced mortality observed in vivo in the mouse. The complex changes in gene expression program after compound exposure could be a direct effect of alterations in a single transcriptional pathway or reflect interactions with multiple gene regulators. Future identification of the specific target(s) of compound action within GAS should provide new insight into the regulation of GAS virulence.

The SK and *fasA* genes are conserved among several pathogenic streptococci that are closely related to GAS, including *S. dysgalactiae* and *S. equi* (11, 26, 40–42). Of note, *S. equi* is the cause of strangles, a serious and often epidemic disease of horses (43). *Fas* also shares homology with the *com* and *blp* operons in *S. pneumoniae* and the *agr* operon in *S. aureus* (25, 44), suggesting that derivatives of CCG-2979 and/or related compounds might also be of potential value in the treatment of other important human and veterinarian pathogens. Finally, in addition to the advantage of milder evolutionary pressure for developing resistance, this class of antimicrobials, acting through an independent mechanism, also offers the potential for synergy with conventional antibiotics (45).

Materials and Methods

Bacterial Strains. UMAA2616 was derived from the GAS M type 1 strain MGAS166 (46). UMAA2616 (originally designated as UMAA2392) contains mutations in the *CovR* and *CovS* gene, leading to enhanced expression of SK and other virulence factors (47). SKKanGAS was generated from UMAA2616 by insertion of a plasmid containing a kanamycin resistance gene (encoding

Table 2. Summary of expression microarray data: dynamics of transcript changes during different bacterial growth phases

Growth phase	Down-regulated			Up-regulated			All changes
	$\downarrow \geq 2$	$\downarrow 1.5\text{--}1.9$	Total down	$\uparrow \leq 2$	$\uparrow 1.5\text{--}1.9$	Total up	
ML	38	95	133	14	19	33	166
LL	56	78	134	10	22	32	166
S	101	113	214	21	41	62	276

ML, midlogarithmic growth phase; LL, transition from logarithmic to stationary phase; S, stationary phase.

Fig. 4. Treatment with CCG-2979 improves survival in an in vivo mouse model for GAS infection. (A) Effect on survival of CCG-2979 [12.7 nmol (5 μ g) per mouse] injected daily for 5 d beginning 24 h after infection of PLGTg+ mice with UMAA2616. Data were pooled from four independent experiments (with GAS inoculation of 8.3, 2.8, 3.6, or 4.0×10^5 cfu/mouse, respectively). A total of 27 mice were represented in the control group treated with vehicle alone (solid line), and 27 mice were represented in the CCG-2979-treated group (dashed line). (B) The same model as in A except that CCG-2979 was injected at a dose of 101.4 nmol (40 μ g) per mouse per day for 4 d. Data were pooled from three independent experiments (with GAS inoculation of 4.22, 3.16, or 1.24×10^6 cfu/mouse, respectively). A total of 22 mice were represented in the control group (solid line), and 19 mice were represented in the CCG-2979-treated group (dashed line). (C) Pooled data from A and B, including all data for CCG-2979. (D) Pooled data for both compounds. CCG-102487 [93.3 nmol (40 μ g) per mouse] was injected daily for 4 d beginning 24 h after infection of PLGTg+ mice with UMAA2616. Data for CCG-102487 (dotted line) are from one experiment (total of 10 mice with GAS inoculation of 4.22×10^6 cfu/mouse). P values were calculated with all pairwise multiple comparison procedures (Bonferroni method).

aminoglycoside phosphotransferase *aphA-3*) driven by the SK gene (*ska*) promoter. Briefly, the kanamycin resistance gene (generated by PCR using primers A and B) (Table S2) was linked to a 1,071-bp GAS genomic sequence upstream of the *ska* coding sequence (generated by PCR using primers C and D) (Table S2) and a 1,004-bp sequence downstream of the *ska* coding sequence (generated by primer E and F) (Table S2), with the final construct verified by direct DNA sequencing. This DNA fragment was then inserted into the multiple cloning sites of the PLZ12(spec) plasmid (36), which replicates and is maintained in Gram-positive bacteria by spectromycin selection. This plasmid was electroporated into the UMAA2616 strain. This newly engineered GAS strain (SKKanGAS) was shown to be kanamycin-resistant, indicating that the *ska* promoter is constitutively active under standard culture conditions. The UMAA2641 strain, generated previously and reported by Khil et al. (15), carries a Ω km-2 cassette constitutively expressing a kanamycin resistance gene (aminoglycoside phosphotransferase *aphA-3*) (48), and it was used as a control for high-throughput screening.

The UMAA2616 strain was cultured in Todd-Hewitt broth containing 0.2% yeast extract (THY) (Difco) supplemented with 100 μ g/mL streptomycin (7). SKKanGAS was grown in the same media also supplemented with 40 μ g/mL kanamycin. Strain MGAS2221 (serotype M1) (49) was grown in THY medium without antibiotics.

High-Throughput, Small-Compound Screen. A diversity-based library of 55,000 small molecules was obtained from the University of Michigan CCG, a collection consisting largely of commercial libraries from ChemBridge, Maybridge, and ChemDiv (www.lsi.umich.edu/ccg/chemical-diversity). Compounds were placed in single wells of a 384-well microtiter plate (#3701; Corning) using the Biomek FX HDR pin tool (Beckman Coulter) at a final concentration of 5–10 μ M in 30 μ L THY medium containing 40 μ g/mL kanamycin. Each compound was screened in duplicate against SKKanGAS and UMAA2641. GAS cultures grown to $OD_{600} = 0.7$ – 0.8 were diluted 12.5- (SKKanGAS) or 25-fold (UMAA2641) into 40 μ g/mL kanamycin THY medium; and 10 μ L this mixture were added into each well using Multidrop (Thermo Labsystems). The difference in GAS dilution compensated for the slower growth in kanamycin of SKKanGAS vs. UMAA2641, probably because of differences in strength between the two promoters driving kanamycin resistance. Positive control wells containing 5 μ g/mL tetracycline, which should completely inhibit GAS growth, were included on each plate. Plates were cultured at 37 $^{\circ}$ C for 16–20 h, and bacteria growth in each well was measured by absorbance at 600 nm.

Selected active compounds from the high-throughput screen were serially diluted (1:2) into 40 μ g/mL kanamycin THY media followed by addition of SKKanGAS or UMAA2641 with the protocol described above to a final DMSO concentration of 1% and final compound concentrations ranging from 3 nM to 100 μ M. Plates were incubated at 37 $^{\circ}$ C for 15–20 h (humidity of 50%), and absorbance was measured at 600 nm in a PHERAstar (BMG Labtech) to estimate the pIC_{50} [$-\log([IC_{50}])$] of bacterial growth inhibition for each compound with kanamycin.

SK Activity Assay. UMAA2616 was inoculated into THY medium, grown at 37 $^{\circ}$ C for 15–20 h, diluted 1:1,000 into fresh THY medium containing varying concentrations of test compounds in a final concentration of 0.1% DMSO, and grown in triplicates at 37 $^{\circ}$ C to an $OD_{600} = 1.0$; 20 μ L culture supernatant were collected after centrifugation at $11,000 \times g$ for 8 min, mixed with 100 μ L PBS, 10 μ L human plasma (Innovative Research), and 10 μ L S2403 (1 mg/mL; Diapharma Group Inc.), and incubated at 37 $^{\circ}$ C for 2 h. SK activity was determined by measurement of cleaved S2403 at OD_{405} and calculated as the percentage of SK activity in a control UMAA2616 culture grown under the same conditions (minus the test compound). Each experiment was performed in triplicate, with six independent experiments for each compound.

Bacterial Growth Assay. UMAA2616 was inoculated into THY medium, grown at 37 $^{\circ}$ C for 15–20 h, diluted 1:100 into fresh THY medium, and cultured with 50 μ M CCG-2979, 50 μ M CCG-102487, or DMSO vehicle alone. OD_{600} was measured at 2, 3, 4, 6, 8, 10, 11, 12, and 24 h to monitor growth. For strain MGAS2221, an overnight culture was diluted 1:100 in prewarmed 50-mL aliquots of THY medium and grown at 37 $^{\circ}$ C in a 5% CO_2 atmosphere. CCG-102487 solubilized in DMSO (final concentration = 20 μ g/mL; 46.7 μ M), or an equivalent volume of DMSO as control was added; bacterial growth was monitored by measuring OD_{600} and viable cell count using the serial dilution method.

Phagocytosis Assay. A stationary phase culture of UMAA2616 was diluted 1:100 into fresh THY medium and grown at 37 $^{\circ}$ C to an OD_{600} of 0.5; 1 mL culture was subject to centrifugation at $10,000 \times g$ for 10 min and washed three times in 1 mL sterile PBS. The bacterial pellet was resuspended in 1 mL PBS and then diluted $1:10^4$ in PBS; 30 μ L GAS suspension were mixed with 90 μ L whole blood collected in 0.2 mg/mL heparin from 6- to 8-wk-old C57BL/6J mice, and test compounds were added to a final concentration of 5 or 50 μ M. Control samples were treated with vehicle (DMSO) alone. After incubation at 37 $^{\circ}$ C with slow rotation for 3 h, 10 μ L were removed and plated on THY plates to determine total cfu. Data were analyzed by one-way ANOVA (Turkey method) using the SigmaStat program (Systat Software).

Microarray Analysis. Microarray analysis was performed using strain MGAS2221 (serotype M1) for comparison with published GAS expression studies as previously described (49). An overnight culture of strain MGAS2221 in THY medium was diluted 1:100 in six prewarmed 50-mL aliquots of THY medium. Three independent cultures were grown in the presence of CCG-102487 (final concentration = 20 μ g/mL; 46.7 μ M), and three cultures were grown in an equivalent volume of vehicle (DMSO) alone; 5-mL samples were collected by centrifugation at $OD_{600} \sim 0.45$ (corresponding to midlogarithmic growth phase), 0.85 (transition from logarithmic to stationary phase), and 3 h after entering stationary phase, and they were stored at -80° C. These 18 samples (three biological replicates $\times$ three growth phases $\times$ two treatments) were processed simultaneously to minimize experimental variance. RNA was isolated, and targets were prepared as described previously (50). Target labeling, hybridization, and data collection were performed

according to instructions provided by the array manufacturer (Affymetrix). Samples were hybridized with a custom GAS array designed for The Methodist Hospital and manufactured by Affymetrix (51).

Chip hybridization data were collected with Affymetrix GeneChip Operating Software (GCOS 1.4). The collected hybridization values were normalized to total hybridization intensity (individual intensities of probes were divided by the sum of all intensities of hybridizing probes). Data derived from three biological replicates were used to calculate mean values. The average values were used to calculate the inhibitor to control transcript ratios. Only genes with ratios above or below 1.5 and P values ≤ 0.05 were included in the results. PartekPro (Partek) and Array Assist (Stratagene) software was used to assess chip quality and chip to chip variability as well as for data mining and visualization.

Murine GAS Infection Model. Six- to eight-week-old human PLG transgenic mice on a C57BL/6J background (backcrossed greater than 10 generations) were injected s.c. with GAS as previously described (7). Briefly, an overnight culture from a single UMAA2616 GAS colony was diluted 1:50 into fresh THY

medium and grown to late log phase ($OD_{600} = 0.75$), and the specific inoculum dose was administered in a 100- μ L volume (diluted in THY medium). The same cultures were plated in parallel on THY plates to determine the exact inoculation titer. After inoculation, mice were injected i.p. daily for 5 d starting 1 d after infection with 100 μ L of 50 μ g/mL test compound in 1.5% DMSO/PBS or vehicle alone. In separate experiments, the test compound dose was increased to 400 μ g/mL delivered daily for 4 d starting 1 d after infection. Mice were monitored for 9 d for morbidity and mortality. Data were analyzed by the Kaplan–Meier method. Survival in different groups was compared using the log-rank test, and all pairwise multiple comparison procedures (Bonferroni method) used the SigmaStat program (Systat Software).

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