A diversity-oriented rhodamine library for wide-spectrum bactericidal agents with low inducible resistance against resistant pathogens

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Antimicrobial resistance is a public health emergency and warrants coordinated global efforts. Challenge is that no alternative molecular platform has been identified for discovery of abundant antimicrobial hit compounds. Xanthene libraries have been screened for bioactive compounds. However, the potentially accessible chemistry space of xanthene dyes is limited by the existing xanthene synthesis. Herein we report a mild one-step synthesis, which permits late-stage introduction of a xanthene moiety onto i.e. natural products, pharmaceuticals, and bioactive compounds and construction of a focused library of rhodamine dyes exhibiting facile functional, topographical and stereochemical diversity. In vitro screening yields 37 analogs with mid-to-high bactericidal activity against WHO priority drug-resistant pathogens. These findings suggest that synthetic dye libraries exhibiting high structural diversity is a feasible chemical space combating antibacterial resistance, to complement the natural sources.
Antibiotics have revolutionized medical practices, have dramatically increased the average life expectancy of humans and are among the greatest medical breakthroughs of the last century. However, antibiotics have only a limited period of clinical-utility before offset by the inevitable emergence of resistance. For example, it took only a few years before resistance toward many antibiotics was discovered, such as penicillin, gentamicin, and ceftazidime. Vancomycin lasted longer before resistance emerged, partly because it was typically used as the last resort. Nevertheless, vancomycin intermediate/resistant pathogens are now widespread. New antibiotics are desperately sought after to fight bacterial resistance. Ironically, large pharmaceutical companies have dramatically shrunken or cut their antibiotic programs. The reasons for this situation are manifold. The golden mine for antibiotics, the secondary metabolites of soil actinomycetes, has run dry after decades of exploitation. An alternative chemical space for abundant antibiotic hit compounds is yet to be identified. Third, antibiotic development has a long expected profit return. As a result, the antibiotic pipeline has been running at an alarmingly slow pace in the past few decades. In particular, among these recently approved few antibiotics, most are actually chemically modified derivatives of the existing classes of drugs, most of which are of natural origin. The resistant strains may readily mutate to resist these analogs if their existing resistance mechanisms do not already exhibit partial cross-effectiveness. The genes encoding resistance to the natural products that are present within the original organisms can also be horizontally transferred to pathogenic microbes enabling resistance to emerge. To prevent the post-antibiotic era becoming reality, alternative chemical space capable of producing abundant antibiotic scaffolds are warranted.

Previously untapped natural sources, i.e. uncultured soil microbes, ocean bacteria, unexpressed metabolites coded by silent operons, and human commensals, have recently been explored and yielded antibiotic compounds. At the same time, synthetic libraries should not be overlooked for antibiotic discovery. Salvarsan for syphilis and prontosil for streptococcus were two classic antimicrobial drugs discovered from the synthetic dye libraries in the early 20th century, and other notable examples are quinolones and the recent addition of oxazolidinones. Despite the fact that target-based antibiotic discovery since the 1990s has been largely unsuccessful, synthetic library of unique chemical space exhibiting high structural complexity and diversity offers opportunities for formulating new paradigms for antibiotic development. Recently, the diversity-oriented fluorescence library approach (DOFLA) has been proven to be a robust method for discovery of bioactive molecules via phenotypic screening. Xanthene dyes, classic small-molecule fluorophores including fluorescein, rhodamine and rhodol, has recently attracted attentions for DOFLA. For example, Chang et al. and other research groups constructed a number of focused libraries based on various fluorescent scaffolds, and further discovered useful imaging agents or fluorescent probes for small-molecule targets, nucleic acids, proteins, lipid droplets, stem-cells and live animals. Burgess et al. constructed a library of mitochondria-targeting rhodamine analogues exhibiting potent anti-proliferative effects toward tumor cells. High structural diversity of dye libraries, the key to its success in the fluorescence library approach, is however the biggest obstacle to overcome. The ubiquitous structure feature of a dye is a two-dimensional conjugated system, to which some simple chemical groups may be attached. Therefore, dyes are typically not endowed with complex three-dimensional features and not ideal for discovery of bioactive compounds via phenotypic screening. Also, the conditions for dye synthesis are usually harsh and installation of chemical groups exhibiting exotic functional, topological and stereochemical features are difficult. Diversity-oriented rhodamine library could not be constructed with the conventional method due to, extensive side-reactions, tedious purification, low yields and most important of all limited scope of compatible substrates. Take the xantheny dye library by Ahn et al. as an example. It is a chemical library of 300+ compounds. However, all follow the general feature, i.e. a xanthen core with a small aromatic group at its C-9. Therefore, the library is not high in terms of diversity. A diversity index of only 3.282 was calculated for this library of rhodamine dyes with Extended-Connectivity FingerPrint (ECFP)38-40, which is a convenient estimate of the structural diversity of a chemical library (vide infra).

We herein report the construction of a diversity-oriented rhodamine library and discovery of potent and wide-spectrum bactericidal agents without inducible resistance. Phenotypic screening of the library yields two promising hit compounds, and RD22 and RD53 were found to be particularly noteworthy through further studies. They inhibit the growth of a wide-spectrum of pathogens, with a potent MIC of 0.5–2 μg mL⁻¹ against the Gram-positive strains, and a MIC of 2–16 μg mL⁻¹ against the Gram-negative strains. They are potent bactericidal agents and pathogens do not readily acquire resistance by genomic mutations toward RD22 and RD53. They exhibit low hemolytic activity with a Lysis20 over 100 μg mL⁻¹. The aforementioned properties of RD22 and RD53, along with other less potent analogs suggest that the rhodamine library is a viable source of antibacterial hit compounds.

Results
Construction of a library with 70 rhodamine analogs. A number of preparative methods are available for xanthene dyes. The earliest synthesis was by von Baeyer in 1871 through the condensation of phthalic anhydride with phenol at high temperature, with Lewis acid or strong proton acid as the catalyst. To accommodate the growing demands for functional xanthene dyes, an alternative mild preparation was introduced by Tsien et al. in 1989, involving nucleophilic addition of a phenyl Grignard (or lithium) reagent with a xanthenone at low temperature. This has enabled the preparation of the library of Ahn et al. Though robust, diversity of the xanthene dyes from this method is still limited by the availability and structural versatility of Grignard reagents. The limitation remains unsolved with the report of two-step Grignard/demethylation method by Yang et al. To address those difficulties with the classic xanthene synthesis, we have developed a mild one-step and high-yielding xanthene synthesis via nucleophilic condensation of a dilithium reagent (Fig. 1a) in the presence of functional xanthene dyes, with exotic substituents at C-9, e.g. phenyls, aryls, alkenyls and acyls (vide infra) (Fig. 1). Shortly after we filed a Chinese invention patent of this method in March 2017, Grimm et al. and Fischer and Sparrow independently developed and reported this method in recent literature. The capability of this method in preparation of rhodamines with substituted phenyl group at C-9 was first examined (Fig. 1b). As a proof-of-concept experiment, the dilithium reagent (1) was reacted with an alkyl benzoate, i.e. methyl benzoate (S1), to furnish RD1 in an 83% yield. Rhodamines with a methyl (RD2), hydroxymethyl (RD3), formal (RD5) or a carboxyl group (RD6) installed at the ortho-position of the bottom phenyl ring was also smoothly prepared in excellent yields by reacting 1 with 2-methyl-benzoate (S2), phthalide (S3), 3-methoxyphthalide (S4) or phthalic anhydride (S6). RD4, rather than RD5, could be...
obtained in an 84% yield if the reaction was quenched with saturated NH₄Cl, rather than dilute HCl solution.

The one-step high-yielding synthesis of RD3 and RD5 shown here is superior to their literature preparations. The ortho fluoro-, chloro- or bromo-substituted methyl benzoate (S7-S9) were compatible with this method to give RD7-RD9 in good to excellent yields. However the attempt with methyl 2-iodobenzoate was complicated with deiodonation leading to the
formation of RD1, presumably via metal-halogen exchange. Rhodamines with hydroxyl, sulphydryl, acetyl, amino and nitro groups (RD10, RD11, RD13, RD14 or RD15) were readily prepared by reacting 1 with benzo[d][1,3]dioxin-4-one (S10), benzo[d][1,3]oxathian-4-one (S11) or benzo[d][1,3]oxazin-4-one (S13), methyl-2-nitrobenzoate (S15) respectively. Synthesis of rhodamine dyes with bulky groups at the ortho position of the bottom phenyl ring is not trivial. This method was powerful in this regard. Reactions between the dilithium reagent 1 and S16, S17, S18, S19 and S20 (Supplementary Figure 1) have produced a series of rhodamine dyes with such bulky groups as phenyll in RD16, RD18, RD19 and RD20, and diphenylphosphoryl in RD17, in a yield ranging from 65 to 70%. The carbonyl of S18 is involved in electronic push-pull system and therefore is not very reactive toward nucleophilic attack. Still, S18 was a viable substrate to react with 1 furnishing a rhodamine (RD18) with an electron-rich phenyl ring at the ortho-position, in a 70% yield. RD20 is a rhodamine modified with four freely rotatable phenyl groups and structurally analogous to an AIE-luminogen47, e.g. tetraphenylethene. Its potential in AIE-based studies warrant further investigations. Rhodamines with phenyl diazo groups (RD21 and RD22) were also successfully prepared in yields over 76%. Unusual structures like RD23 or RD24, expected to exhibit high molar absorptivity and fluorescence brightness were successfully obtained, in good yields of 56% and 49%, respectively.

Onto the C-9 position of the rhodamine core can be installed aryl groups other than phenyl, e.g. polyaromatic moieties, heterocyclic aromatic moieties and the exotic sandwich/half-sandwich complexes (Fig. 1c). Rhodamines with naphthalene (RD25, RD26) or anthracene (RD27, RD28) were prepared from reactions of 1 with the corresponding ester or anhydride in good isolated yields (typically over 75%). Esters of pyridine, quinoline, furan, thiophene, pyridazine, pyrimidine, pyrazine, imidazole, oxazole, and thiazole (Supplementary Figure 1) could also react with the dilithium reagent 1 to prepare the corresponding rhodamine dyes (RD29–RD38). With the methyl ester of ferrocene, we prepared RD39, in which the ferrocene moiety is directly bonded to the C-9 position of the rhodamine core. We further showed that the half-sandwich complex could be directly attached to the rhodamine core (RD40). The potential applications of RD40 in sensing, imaging and catalysis are attractive. Substrates bearing two ester moieties could be used to template multiple rhodamine cores, to give RD41.

Alkenyl groups can also be installed onto the C-9 position of a rhodamine core (Fig. 1d). The double bond in such rhodamine dyes exhibits facile chemistry and may be useful in site-specific labeling48, chemosensing of nucleophiles49 and reactive oxygen species50 and optical switching51. With the ester of a coumarin, a rhodamine-coumarin dye pair (RD42) was synthesized as one example of this group of rhodamine dyes. Reactions between the dilithium reagent 1 and a range of substrates like maleic anhydride, cinnamates, or coumarins (Supplementary Figure 1) were all capable of furnishing such alkenyl substituted rhodamine dyes, i.e. RD43–RD47. We note that the double bond of RD43 adopts cis-conformation, as suggested by a coupling constant of 5.4 Hz between the two alkyl $^1$H's. The double bonds in RD44–RD47 are all in trans-conformation, regardless of the cis- or trans-conformation of the double bond in the corresponding starting material (S44–S47, Supplementary Figure 1).

Alkyl esters, lactones and anhydrides are all feasible substrates for synthesizing rhodamine dyes and diverse range of complex alkyl groups are also conveniently installed to the C-9 of the rhodamine core (RD48–RD59) (Fig. 1e).

Till this point, we have demonstrated that this rhodamine synthesis is convenient, powerful and reliable. The range of substrates compatible for this method is exceedingly broad, including esters, anhydride, lactone, aryl lactam, and potentially more. To further promote the scope of this rhodamine synthesis, we are particularly interested in the possibility of using ester-/lactone-/anhydride-containing bioactive molecules, e.g. pharmaceuticals and pesticides (S60–S69, Supplementary Figure 1), in rhodamine synthesis. The structures of such compounds are typically complex and there were no attempts to use them in dye synthesis. Though probably unexpected to the field, many of such compounds, e.g. telmisartan methyl ester (S60), noscapine (S61), adalapene (S62), a precursor of candesartan (S63), etomidate (S64), the methyl ester of flumazenil (S65), levofloxacin methyl ester (S66), vinpocetine (S67), methoxsalen (S68), ethyl chrysanthemate (S69) were indeed viable substrates for this rhodamine synthesis (Fig. 1f).

This method is also suitable for preparation of an oxazine dye RD70 (Fig. 1g) from isoamyl nitrite, which differs from its rhodamine analog in that it bears a nitrogen atom rather than a methane carbon.

**Diversity index.** The extended-connectivity fingerprint (ECFP) method was employed to assay the diversity of our library because of its wide-acceptance in drug discovery and has been integrated into various commercial softwares. ECFPs encode each atom and its molecular environment within a circle with a diameter of varying radius of chemical bonds. Depending on the chosen bond radius, different numbers of structural features having different sizes are produced. In this study, we introduce the definition of the total number of fingerprint features as a quantitative measure of the structural diversity of a library. We calculated the specified fingerprint for each molecule using ECFP$_6$ and counted the total number of unique fingerprint features collected over all the molecules. Then the Diversity Number Finger Print Features (DNFPF value) was defined as the total number of fingerprint features divided by the number of molecules. A DNFPF value of 18,875 was calculated for the obtained library of rhodamine dyes (RD1–RD69), while a DNFPF value of 3.228 was found for the Ahn’s library$^{37}$ of rhodamine dyes (Supplementary Table 6). This verified that the synthetic method presented herein is feasible for construction of a rhodamine library with much enhanced structural diversity.

**Fluorescence properties.** The fluorescence properties of RD1–RD70 were studied predominantly in PBS (50 mM, pH = 7.4 containing 1% DMSO) with UV-Vis absorption and fluorescence spectroscopies (Fig. 2 and Supplementary Table 1). Other solvents were not tested because we are typically concerned with the potentials of this library in bio-oriented applications. The maximal absorption wavelengths of rhodamine dyes with alkyl groups (RD48–RD59) at C-9 typically absorb at shorter wavelength region of 545–560 nm and ones substituted with heteroaryl groups (RD25–RD41) absorb at longer wavelength region of 551–585 nm, while others absorb in the range of 560–570 nm. Most dyes exhibit a small Stokes shift of 30 nm, e.g. 34 nm for RD31, 41 nm for RD33, 37 nm for RD35, and 34 nm for RD41, 32 nm for RD47, 39 nm for RD53, 35 nm for RD56, 42 nm for RD59. The fluorescence brightness of many dyes of this library is comparable or higher than that of the rhodamine B (RD6). They are useful for cell imaging-based applications (Supplementary Figure 72–131). Many others are much less fluorescent. Dim dyes may be used as fluorescent probes, in case their fluorescence may be chemospecifically turned on. For example, we have found that RD17 can respond to nitroxy (HNO) and induce a $\Delta a$ 5 fold fluorescence turn-on.
Antibacterial studies of the rhodamine library. The anti-
microbial activity of (RD1-RD70) were preliminarily evaluated
against two drug-resistant bacteria, which were a strain of Gram-
positive methicillin-resistant Staphylococcus aureus (MRSA,
ATCC43300) and a strain of Gram-negative Acinetobacter baum-
mannii (ATCC19606). The minimum inhibitory concentrations
(MIC) of each compound were measured using the standard CLSI
broth protocol (Fig. 3, Supplementary Table 2). Typically, anti-
microbial potency of a compound is considered mid-to-high if its
MIC is 8 μg mL⁻¹ or lower. Out of the total 70 analogs, 37 were
found to exhibit a MIC of 8 μg mL⁻¹ or lower against MRSA, and
5 against A. baumannii. This is a result much to our delight as
well as to our surprise considering the relentless yet largely futile
efforts that the community has dedicated to the quest for a
molecular platform for discovery of abundant compounds with
antimicrobial activity. Further analysis revealed that these
bioactive analogs are quite scattered in terms of the nature of
substrate at the C-9 position of the rhodamine core. Among
these bioactive analogs, 14 analogs bear a phenyl group at C-9
position, 12 a heteroaromatic moeity, 6 an alkyl and 5 an alkyl
group. Presumably, the rhodamine core is the pharmacophore
and the substituent at the C-9 playing a subsidiary, yet critical
role. These results strongly suggested that this diverse-oriented
rhodamine library is a viable platform for discovery of potent
antimicrobial agents.

Though all these 37 + 5 compounds with mid-to-high anti-
bacterial activity worth further pursuing, we prioritized the ones
with a higher activity for in-depth study of their antibiotic
spectrum (Table 1). Those compounds with the lowest MIC’s
against ATCC43300 (RD1/15/22/31/45/46/53) or ATCC19606
(RD12/22/44/45/53) were chosen for further screening against
three more Gram-positive bacteria, including the ATCC25923
(methicillin-sensitive Staphylococcus aureus, MSSA), ATCC51299
(vancomycin-resistant Enterococcus faecalis, VRE), and
ATCC29212 (vancomycin-sensitive Enterococcus faecalis, VSE),
and three Gram-negative bacteria, including ATCC13883 (Klebsiella
pneumoniae), ATCC25922 (Escherichia coli) and ATCC27853 (Pseudomonas aeruginosa) (Table 1). RD22/46/53
showed potential broad-spectrum Gram-positive antibiotic activity
with a low MIC of 0.5–2 μg mL⁻¹, while RD1/15/31/45 were
much less active toward E. faecalis compared to their activity
against S. aureus. RD22/44/53, but not RD12/45, also exhibited
antibiotic activity toward Gram-negative bacteria other than
ATCC19606, with a relatively higher MIC of 8–32 μg mL⁻¹.
From this study, RD22/53 stood out as the two most attractive
hits. First, their MIC’s against MRSA is 1 μg mL⁻¹, the lowest
among this shortlist, and comparable or even lower than those of
vancomycin and linezolid (Table 1), the last resorts for MRSA
infections. Second, they were also equally potent toward both
vancomycin-sensitive and vancomycin–resistant E. faecalis with
a MIC of 2 μg mL⁻¹. Third, they were also active toward various
clinically significant Gram-negative bacteria with MIC of 4–16 μg
mL⁻¹. These encouraging preliminary results promoted us to
explore the antibiotic profile of RD22/53 in a greater depth.
The antibiotic spectrum of RD22 and RD53 were further
checked against a panel of standard reference and clinical-isolated
Gram-positive and Gram-negative strains, which encompass
most of the 12 deadliest drug-resistant bacteria classified by
the World Health Organisation (WHO) (Table 2). The
Gram-positive stains include S. aureus (MRSA, MSSA), E. faecalis and
E. faecium (VRE and VSE), S. epidermidis, S. pyogenes, H. pylori,
S. pneumonia, while the tested Gram-negative stains are A.
baumannii, P. aeruginosa, E. coli, K. pneumonia, and S. flexneri.
RD53 exhibited a potent MIC of 0.25–2 μg mL⁻¹ toward all the
aforementioned Gram-positive strains, regardless of the drug-
resistance profile of these bacteria. The potency of RD53 toward
the Gram-negative strains was typically lower compared to the
Gram-positive strains. For example, a MIC of 4 μg mL⁻¹ was
observed for A. baumannii, and 16 μg mL⁻¹ for P. aeruginosa,
2–8 μg mL⁻¹ for various strains of E. coli, 4–16 μg mL⁻¹ for K.
Pneumoniae and 1 μg mL⁻¹ for Shigella flexneri. RD53 was not
only found to be effective toward standard reference strains, but

![Fig. 2](image_url) Fluorescence properties of the rhodamine library in PBS buffer. The y-
axis is the brightness, which is expressed as log (ε × Φ) of a given
rhodamine dye and the x-axis is the absorption maximum. RD23 and RD24
were tested in EtOH (Supplementary Figure 70) and RD28 may scavenge \( ^1 \text{O}_2 \) and
produce a fluorescence turn-on of ca. 14 fold (Supplementary Figure 71).

![Fig. 3](image_url) Activity of RD1-RD70 against MRSA and Acinetobacter baumannii.
The MIC (μg mL⁻¹) of each compound was measured by the standard CLSI
broth protocol.
elaboration. However, in some serious clinical situations, such as endocarditis, meningitis, osteomyelitis, neutropenia, bactericidal action is necessary because of the reduced rates of metabolism and cell division, the poor immunologic competence or the poor drug penetration\textsuperscript{35}. The bactericidal activity of RD22 and RD53 were compared to multiple first-line antibiotics using the time-kill assay (Fig. 4). Methicillin-resistant \textit{S. aureus}, Vancomycin-resistant \textit{E. faecalis}, and polymyxin E-resistant \textit{A. baumannii} were chosen as the tested Gram-positive and Gram-negative strains, respectively. MRSA (ATCC43300) were grown to early exponential phase and challenged with 10× MIC of vancomycin, linezolid, tigecycline, respectively (Fig. 4a). In the first 4 h, bacteria concentration remained steady in the presence of vancomycin and linezolid and then gradually dropped by two-three orders of magnitude within 24 h. Tigecycline exhibited a higher degree of bactericidal activity than vancomycin and linezolid. In comparison, RD22 (Fig. 4b) and RD53 (Fig. 4c) were more potent bactericidal agents than vancomycin, linezolid or tigecycline. Bacteria challenged by as low as 2.5× MIC lowered the bacteria concentration by four order of magnitude of bacteria within as short as 2 h. RD22 and RD53 also exhibits bactericidal activities to VRE (ATCC51299) (Fig. 4e, f). They lowered the concentration of VRE by over 4 orders of magnitude within 2 h by (5–10) MIC. In comparison, linezolid, tigecycline did not exhibit strong bactericidal activity and bacterial concentration dropped by less than 2 orders of magnitude within 24 h. Daptomycin was able to reduce the cell concentration by over 4 orders of magnitude within 8 h (Fig. 4d). Polymyxin E-resistant \textit{A. baumannii} (MIC = 256 μg mL\textsuperscript{-1}, stored in the lab) was obtained by serial passages of cells at sub-MIC concentration of polymyxin E during resistance acquisition of pathogens. RD53 at 10× MIC was able to reduce the cell concentration by over 4 orders of magnitude within 6 h (Fig. 4i) while at the same dose of tigecycline and RD22 reduced the cell concentration by around 3 orders of magnitude (Fig. 4g, h). And RD53 at 8× MIC could also reduce the cell concentration of \textit{H. pylori} by more than 4 orders of magnitude within 2 h (Supplementary Figure 132). So, the time-kill assay proved that RD22 and RD53 are potent bactericidal agents. They can potentially complement bacteriostatic antibiotics in serious bacterial infections to hosts, whose immune response is compromised.

Resistance studies of RD22 and RD53. Ease of resistance acquisition toward RD22 and RD53 was evaluated using MRSA, VRE and \textit{A. baumannii} as tested bacteria (Fig. 5). Serial passages of MRSA in the presence of sub-MIC levels of levofloxacin over a

Table 1 Antimicrobial activity of selected RD dyes against four Gram-positive and four Gram-negative bacteria

| MIC (μg mL\textsuperscript{-1}) | ATCC43300 | ATCC25923 | ATCC51299 | ATCC29212 | ATCC19606 | ATCC13883 | ATCC25922 | ATCC27853 |
|-----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| RD1             | 1         | 0.5       | 16        | 16        | /         | /         | /         | /         |
| RD12            | /         | /         | /         | /         | /         | /         | /         | /         |
| RD15            | 1         | 0.5       | 32        | 16        | /         | /         | /         | /         |
| RD22            | 1         | 0.5       | 2         | 2         | /         | /         | /         | /         |
| RD31            | 0.5       | 0.5       | 16        | 16        | /         | /         | /         | /         |
| RD44            | /         | /         | /         | /         | /         | /         | /         | /         |
| RD45            | 1         | 1         | 8         | 4         | /         | /         | /         | /         |
| RD46            | 1         | 1         | 2         | 2         | /         | /         | /         | /         |
| RD53            | 1         | 1         | 2         | 2         | 4         | 16        | 8         | 16        |
| vancomycin      | 2         | 2         | /         | /         | /         | /         | /         | /         |
| linezolid       | 1         | 2         | /         | /         | /         | /         | /         | /         |
| polymyxin E     | /         | /         | /         | /         | /         | /         | /         | /         |
| tigecycline     | 2         | 1         | /         | /         | /         | /         | /         | /         |
| daptomycin      | 2         | /         | 4         | /         | /         | /         | /         | /         |

Table 2 Antibacterial activity of RD22 and RD53 against a panel of Gram-positive and Gram-negative pathogens

| Organism          | MIC*                  | RD22 | RD53 |
|-------------------|-----------------------|------|------|
| Gram+             |                       |      |      |
| \textit{S. aureus} | ATCC25923 (MSSA\textsuperscript{a}) | 1    | 1    |
|                   | CMCC26003 (MSSA\textsuperscript{a}) | 1    | 0.5  |
|                   | ATCC43300 (MRSA\textsuperscript{a}) | 10   | 0.25 |
|                   | 10 Clinical isolates (MRSA\textsuperscript{a}) | 0.25 | 0.25–2 |
| \textit{E. faecalis} | ATCC29212 (VSE\textsuperscript{b}) | 2    | 2    |
|                   | ATCC51299 (VRE\textsuperscript{b}) | 2    | 2    |
|                   | 5 Clinical isolates (VSE\textsuperscript{b}) | 1    | 1    |
| \textit{E. faecium} | ATCC35667 | 1    | 2    |
|                   | 5 Clinical isolates (VRE\textsuperscript{b}) | 1    | 1    |
|                   | 3 Clinical isolates (VSE\textsuperscript{b}) | 1    | 0.5–1 |
| \textit{S. epidermidis} | CMCC26069 (MSSE\textsuperscript{c}) | 2    | 1    |
|                   | 2 Clinical isolates (MSSE\textsuperscript{c}) | 1    | 1    |
|                   | 1 Clinical isolates (MRSE\textsuperscript{c}) | 1    | 1    |
| \textit{S. pyogenes} | CMCC32006 | 3    | 1    |
|                   | 3 Clinical isolates | 0.5 | 2–4  |
| \textit{H. pylori} | Sydney Strain 1 | n.a. | 0.5  |
| Gram-             |                       |      |      |
| \textit{A. baumannii} | ATCC19606 | 4    | 4    |
| \textit{P. aeruginosa} | ATCC27853 | 16   | 16   |
| \textit{E. coli} | ATCC25922 | 16   | 8    |
|                   | 9 Clinical isolates (ESBL\textsuperscript{d}) | 2–4 | 2–4  |
|                   | 4 Clinical isolates (ESBL\textsuperscript{d}) | 4    | 4–8  |
| \textit{K. pneumoniae} | ATCC13883 | 16   | 16   |
|                   | 3 Clinical isolates (ESBL\textsuperscript{d}) | 4    | 4–8  |
|                   | 4 Clinical isolates (CRE\textsuperscript{e}) | 4–8 | 8–16 |
| \textit{S. flexneri} | ATCC15081 | 2    | 1    |

*MIC’s are given in unit of μg mL\textsuperscript{-1}.
\textsuperscript{a}MSSA (or MRSA): methicillin-sensitive (or resistant) \textit{Staphylococcus aureus}.
\textsuperscript{b}VSE (or VRE): vancomycin sensitive (or resistant) Enterococcus.
\textsuperscript{c}MSSE (or MRSE): methicillin-sensitive (or resistant) \textit{Staphylococcus} \textit{epidermidis}.
\textsuperscript{d}ESBL: extended spectrum beta-lactamases.
\textsuperscript{e}CRE: carbapenem-resistant Enterobacteriaceae.

also effective toward a broad range of clinically isolated, difficult-to-treat, multidrug-resistant bacteria, including \textit{S. aureus} (MRSA, 10 strains), \textit{E. faecalis} (VSE, 5 strains), \textit{E. faecium} (VRE, 5 strains; VSE, 3 strains), \textit{S. epidermidis} (MSSE, 2 strains; MRSE, 1 strain), \textit{S. pyogenes} (3 strains), \textit{S. pneumoniaia} (3 strains), \textit{E. coli} (ESBL\textsuperscript{+}, 9 strains; ESBL\textsuperscript{−}, 4 strains), \textit{K. pneumoniae} (ESBL\textsuperscript{+}, 3 strains, ESBL\textsuperscript{−}, 4 strains, and CRE, 4 strains). Compared to RD53, RD22 displayed equally potent antimicrobial activity against all the aforementioned standard reference Gram-positive and Gram-negative type bacteria and clinical isolates as well.

The clinical significance of the first-line bacteriostatic antibiotics, e.g., vancomycin, linezolid, et al., warrants no further
Fig. 4 Time-dependent killing of pathogens by **RD22** or **RD53** compared to clinical antibiotics. MRSA (ATCC43300) challenged with **a** vancomycin, linezolid, tigecycline; **b** RD22; **c** RD53. VRE (ATCC51299) challenged with **d** vancomycin, linezolid, tigecycline, daptomycin; **e** RD22; **f** RD53. Polymyxin E-resistant Acinetobacter baumannii, challenged with **g** polymyxin E, tigecycline **h** RD22; **i** RD53. Pathogens were grown to early exponential phase and challenged with **RD22/53** or clinical antibiotics. Data are representative of three independent experiments. Error bars indicate s.d.

Fig. 5 Resistance acquisition of pathogens during serial passaging in sub-MIC levels of antimicrobials. The y axis is the fold change of MIC. **a** MRSA (ATCC43300) towards **RD22** (MIC = 1 μg mL⁻¹, tested up to 2 × MIC), **RD53** (MIC = 1 μg mL⁻¹, tested up to 1 × MIC), levofloxacin (MIC = 0.5 μg mL⁻¹, tested up to 128 × MIC), and vancomycin (MIC = 2 μg mL⁻¹, tested up to 2 × MIC). **b** VRE (ATCC51299) toward **RD22** (MIC = 2 μg mL⁻¹, tested up to 1 × MIC), **RD53** (MIC = 1 μg mL⁻¹, tested up to 2 × MIC), linezolid (LZD, MIC = 2 μg mL⁻¹, tested up to 64 × MIC), ciprofloxacin (CIP, MIC = 1 μg mL⁻¹, tested up to 32 × MIC), and daptomycin (DAP, MIC = 4 μg mL⁻¹, tested up to 128 × MIC). **c** A. baumannii (ATCC19606) toward **RD22** (MIC = 4 μg mL⁻¹, tested up to 8 × MIC), **RD53** (MIC = 4 μg mL⁻¹, tested up to 32 × MIC), polymyxin E (PmE, MIC = 1 μg mL⁻¹, tested up to 256 × MIC), tigecycline (TGC, MIC = 2 μg mL⁻¹, tested up to 32 × MIC), and ciprofloxacin (CIP, MIC = 1 μg mL⁻¹, tested up to 256 × MIC), meropenem (MEM, MIC = 1 μg mL⁻¹, tested up to 64 × MIC). Data are representative of three independent experiments. Error bars indicate s.d.
period of 20 days raised the MIC by 128 folds, i.e. rendering the strain highly resistant to levofloxacin. In comparison, MRSA did not develop resistance toward vancomycin, exactly the reason why vancomycin serves as the last-line antibiotic against MRSA. It is very interesting to note that MRSA also did not acquire resistance toward tigecycline (TGC), ciprofloxacin (CIP), polymyxin E, and polymyxin E (PmE), levofloxacin (LEV), and ampicillin-sulbactam (SAM), remains unchanged compared to ATCC19606, suggesting the lack of cross-resistance between RD53 and these antibiotics (Supplementary Table 9). Third, selective pressure on antibacterial targets could also be measured by genomic profile of mutational resistant bacteria. So, the genomic analysis was carried out for clues on the potential mechanism of action of RD53. Comparative analysis revealed the existence of eight nonsynonymous single nucleotide variants (SNV) and one nonframeshift deletion (Table 3), which belong to resistance/tolerance-associated genes encoding efflux pumps, metabolism, DNA replication or transcriptional regulation related proteins. It is interesting to find the mutation in rsmJ which encodes 16S rRNA (guanine1516 - N2) – methyltransferase. Notably, the mutation in 16S RNA methyltransferase has been shown to cause kasugamycin resistance in E. coli. These clues together have formulated a solid framework for further investigation of the mechanism of action of RD53.

**Structure-activity relationship investigation.** The structure-activity relationship of these rhodamine-type antibiotic compounds was examined. Out of the 70 members of this rhodamine library, 37 compounds exhibit antibiotic activity of varying potency (Fig. 3). This fact alone strongly suggested that their common structural motif is the pharmacologically active motif, which is the rhodamine core. To verify this hypothesis, we have prepared a few closely related analogs (C1-2, and RD71-74, Fig. 7). First, C1 and C2 differ from the RD53 in that C1 misses the oxygen atom of the rhodamine core compared to RD53, and C2 has one more aniline moiety removed compared to C1. Both C1 and C2 exhibited no appreciable antibiotic activity with their MIC’s higher than 64 µg mL⁻¹ toward both ATCC43300 and ATCC19606 (Fig. 7a). Second, the peripheral group of the rhodamine core or the adamantane moiety could be modified without inhibiting the antibiotic activity as long as the rhodamine core remains intact, as showcased with RD71-74 (Fig. 7b, c). The aforementioned hypothesis was further supported by the following experimental results that none of the starting materials (S1-69) for preparations of these rhodamine analogs (RD1-RD69) display antibiotic activity (MIC > 64 µg mL⁻¹) (Supplementary Table 3). This reiterates the necessity of the rhodamine core for antibiotic activity. Additional support to this hypothesis comes from the ten compounds listed in Fig. 7d. These compounds predominantly adopt the ring-closed form and therefore they do not actually have the rhodamine motif. And indeed, none of them have been found to exhibit antibiotic activity during the phenotypic screening. The discovery of the above structure-activity relationship is important as it provides a general guideline for further structural modification, if warranted in the future drug-development endeavors.

**Discussion**

Diversity-oriented synthesis of xanthene library attracted attention for discovery of original bioactive compounds through phenotypic screening. This has promoted search for a mild xanthene synthesis allowing facile installation of functional, topographical and stereochemical diversity. We have devised a mild, one-step, high-yielding and readily scalable xanthene synthesis, by reacting a rationally constructed dilithium species (Fig. 5a). A separate experiment, VRE developed resistance toward linezolid, ciprofloxacin and daptomycin by serial passages in 20 days, with a raise of MIC’s by 32–128 folds while no obvious resistance was observed with RD22 and RD53 by VRE (Fig. 5b). The Gram-negative A. baumannii readily acquired resistance by genomic mutations toward polymyxin E and ciprofloxacin with a 256 enhancement of MIC in as short as 7 days. Bacteria did develop resistance toward meropenem, tigecycline, RD22 and RD53 as well, but to a much reduced degree, i.e. 64-, 32-, 8-, and 32-fold enhancement of MIC within 20 days, respectively (Fig. 5c). It has become evident that either Gram-positive or Gram-negative pathogens were found not to readily acquire resistance by genomic mutations toward RD22 and RD53. Analogously, E. coli, E. faecalis and S. aureus were found not to acquire resistance toward RD53 (Supplementary Figure 133). Development of resistance toward an antibiotic by bacteria is an evolutionary inevitability and thereby imposes a limit on the lifespan of any given antibiotic. And resistance was involved in clinical failure of some antibiotic administration regimes. This is especially evident because the antibiotic levels at some points during treatment are in the sub-inhibitory range. Therefore, the low tendency of resistance acquisition toward RD22 and RD53 by various pathogens greatly enhances their potentials for further drug development.
with alkyl/aryl/alkenyl substituted esters, anhydrides, lactones and even some lactams and synthesized a focused library of xanthene dyes with 70 members. Through in vitro screening against various multi-drug-resistant pathogens, we found that it is a rich source of hit compounds exhibiting potent bactericidal activity. Particularly, RD22 and RD53 are two antibacterial hits exhibiting favorable properties for further drug development. First, the growth of a wide-spectrum of Gram-positive and Gram-negative pathogens was inhibited by RD22 and RD53 with a low MIC. They are a potent bactericidal agent and a low dose at (2.5–10) × MIC could reduce the concentration of methillin-resistant S. aureus, vancomycin-resistant E. faecalis, polymyxin E resistant A. baumannii by 4 orders of magnitude within ca. 4 h, more quickly than the current first-line and last-resort antibiotics, e.g. vancomycin, linezolid, tigecycline, and dapto mycin. While the pathogens readily acquire resistance toward these existing antibiotics, exposing the cells toward sub-MIC concentration of RD22 and RD53 did not readily induce emergence of resistance. Fourth, they have a large therapeutic index of over 100, which is defined by the Lysis20/MIC. Our work is reminiscent of the discovery of Prontosil, the first antibiotic, from an azo dye library by Gerhard Domagk. The successful treatment of streptococcus infection of her daughter foreran what was later known as the Golden Age of Antibiotics. With this manuscript, we want to share with the community that dye libraries of high structural diversity could potentially be that sought-after chemical space of further drug development.

Methods

Construction and characterization of the rhodamine library. Details of synthesis and characterization of RD1 RD74, C1-C2 employed in this manuscript were included in the Supplementary Methods section (Supplementary Figure 383-393). Structures of substrates (S1 S69) can be found in Supplementary Figure 1. General procedures B was illustrated as the general synthetic route for the preparation of rhodamine RD1-72 via nucleophilic condensation of a dilithium reagent 1 with various substrates S1-S70. The UV-Vis absorption and fluorescence emission spectra in pH = 7.4 PBS with 1% DMSO was shown in Supplementary Figures 2-69, and spectral properties were summarized in Supplementary Table 1. For 1H-13C-NMR and MS spectra see Supplementary Figures 135-382.

Bacterial cell culturing. The following bacterial strains were used: Staphylococcus aureus ATCC25923 (American type culture collection, Beijing Zhongyuan Ltd., cat. no. 25923), CCMM26003 (National Center for Medical Culture Collections, cat. no. 26003), ATCC43300 (American type culture collection, Beijing Zhongyuan Ltd., cat no. 43300), Enterococcus faecalis ATCC29212 (American type culture collection, Beijing Zhongyuan Ltd., cat. no. 29212), ATCC31299 (American type culture collection, Beijing Zhongyuan Ltd., cat. no. 31299), Enterococcus faecium ATCC53667 (American type culture collection, Beijing Zhongyuan Ltd., cat. no. 35667), Staphylococcus epidermidis CCMM26069 (National Center for Medical Culture Collections, cat. no. 26069).

| Mutationa | Gene nameb | Definition | Amino acid change |
|-----------|------------|------------|------------------|
| G → C    | *kup*      | KUP system potassium uptake protein | A373P |
| A → T    | *mexB*     | multidrug efflux pump | S617T |
| A → G    | *mexB*     | multidrug efflux pump | L149P |
| G → T    | *trkH*     | *trkG*, *ktrB* | S14R |
| G → A    | *nrdA*     | NAD-dependent deacetylase | A14T |
| C → T    | *rsml*     | 16S rRNA (guanine1516-N2)-methyltransferase | A228T |
| C → T    | *hisD*     | histidinol dehydrogenase | V257A |
| G → A    | *etk-wzc*  | tyrosine-protein kinase Etk/Wzc | D569N |

*NS* indicates the change in *kup* gene.

Table 3 Mutations present in RD53-resistant A. baumannii

| SNPs | Gene namea | Definition | Amino acid change |
|------|------------|------------|------------------|
| C → A | *slt*      | Slt methyltransferase | Q394E |
| C → A | *ipl*      | Ipl methyltransferase | Q461E |
| G → A | *ggt*      | Ggt phosphomonoesterase | G591E |
| C → A | *sln*      | Sln periplasmic protein | Q598A |
| G → A | *sra*      | Sra efflux pump | G604A |
| G → A | *srl*      | Srl efflux pump | G604A |

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Fig. 7 Structure-activity relationship investigation of the rhodamine library. 

- **a**: RD53 analogs without the rhodamine core;
- **b**: RD53 analogs with the adamantane moiety modified;
- **c**: RD53 analogs with the diethylamino group modified.

**d**: Rhodamine dyes mainly adopted the ring-close forms exhibit no antibacterial activity against ATCC43300 or ATCC19606, with MIC > 64 μg mL⁻¹.
insertions/deletions (InDel) with the GATK (V3.4.6) software. Annotation for
with a quality lower than 20 were removed using Cutadapt (V1.9.1). The clean data
were employed to analyze genomic structure variation.

Data availability
The data that support the findings of this study are available from the corre-
sponding authors on request.

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Author contributions
Y.Y., D.C. and X.Q. conceived the projects. X.L. designed the synthetic method, synthesis RD1–72 and acquired the UV-Vis absorption and fluorescence properties. Y.X., L.G., Z.L. and T.H. synthesized RD73–74 and C1–C2. L.Q. measured the MICs of standardized bacteria, collected the dose-kil curve and carried out the induced resistance test and did the SEM imaging of bacteria. L.S., X.D., J.B. and J.W. contributed to phenotypic screenings. X.L., Y.T., Y.Z., J.Z. and W.Z. did the confocal imaging of mammalian cells. X.W. and H.L. calculated the diversity index. F.H. measured the MICs of the clinical strains. All authors contributed to manuscript preparations.

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