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Synergy of Non-antibiotic Drugs and Pyrimidinethiol on Gold Nanoparticles against Superbugs

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Supporting Information

ABSTRACT: Co-presenting non-antibiotic drugs and pyrimidinethiol on gold nanoparticles (NPs) can generate broad-spectrum antibacterial and bactericidal activities against superbugs. Dimethylbiguanide (metformin), an anti-hyperglycemic drug, shows the best enhanced activity via increasing the ability to compromise bacterial cell walls. Synergistic effects are also reflected in the eradicating biofilm cells. Our findings suggest a large chemical space to develop new antibacterial materials to treat superbugs.

We have reported amino-substituted pyrimidinethiol, an analogue of tRNA cytosine in *Escherichia coli* (*E. coli*) to modify gold NPs in order to kill Gram-negative bacteria.[1] Here we explore synergistic effects of non-antibiotic drugs with gold NPs to deter bacterial resistance with broad spectra. Unlike combinatorial strategies for common antibiotics, synergistic effects for NPs cannot be achieved by simply mixing candidate compounds with NPs, but occur only when both candidate compounds and pyrimidinethiol were presented as co-ligands on NPs.

Multidrug-resistant superbugs emerge worldwide at an unprecedented speed and lead to a public health threat, which include Gram-positive vancomycin-resistant *Enterococcus faecium* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and Gram-negative multidrug-resistant (MDR) *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species, abbreviated as "ESKAPE".[2] Approaches targeting cell membrane were reported to be effective in treating superbugs, such as cationic, amphiphilic polycarbonate nanoparticles,[3] cationic lipoglycopeptides,[4] inhibitors of lipopolysaccharide transport,[5] proteolysis agents of periplasmic proteins,[6] peptidoglycan-recognizing proteins,[7] inhibitor of ATPase,[8] and combination of non-antibiotic drugs with antibiotics.[9] Screens of synergistic drugs from non-antibiotic drug library offer an expanse of use of existing antibiotics. However, the screening efficiency is limited because of the low output where four active compounds were obtained from a library with 1057 compounds.[9a] It is well known that most of the above-mentioned strategies include cationic units, such as acetylcholine and guanidine groups, which can interact with the cell membrane. Thus, we explored synergistic effects of non-antibiotic compounds composed of such amine groups as co-ligands on gold NPs.

Compared with conventional antibiotic molecules, the inherent features of gold NPs with advantages of large surface area, straightforward surface modification by thiols and amines,[10] not being the substrate of bacterial efflux pumps, and the safety approved by U.S. FDA to treat cancer[11] all allow gold NPs to be useful as antibacterial agents.[1,12] We have developed a strategy to fight against MDR Gram-negative bacteria by presenting inactive small molecules, such as 4,6-diamino-2-pyrimidinethiol (DAPT or D) on gold NPs as an antibacterial agent,[1] which act on bacteria via dissipating membrane potential and inhibiting energy metabolism.[13] However, these NPs show less activity on Gram-positive bacteria, possibly due to the thick peptidoglycan of such bacteria.[14] Other researchers have shown that various thiols as mixed ligands of gold NPs showed activities at high concentrations.[15] Hydrophobic, cationic thiol-capped 2-nm gold NPs are proved to interact with bacteria[16] and kill Gram-positive bacteria but inactive to Gram-negative bacteria.[17] Here, we report that a class of non-antibiotic amines (NAA, most of them are FDA-approved drugs) show synergistic antibacterial effects with DAPT when presented as co-ligands on gold NPs against superbugs with broad spectra, including the notorious MDR bacteria "ESKAPE".

We chose guanidine (G), 1,1-dimethylbiguanide (DMB), 1-(3-chlorophenyl)biguanide (CPB), chloroquine diphosphate (CQ), acetylcholine chloride (ACh), and melamine (Mel) to modify gold NPs, which are used alone or mixed with DAPT as co-ligands to cap gold NPs (abbreviated as Au_NAA or Au_D/NAA, Figure 1). G, a drug used to treat muscle weakness,[18] is often used as a cell-permeability moiety of cell-penetrating peptides,[19] dendrimers,[20] and antibiotics.[21] Derived from guanidine, DMB is an anti-hyperglycemic drug (metformin) and CPB is an agonist of serotonin type 3 receptor.[22] Chloroquine is an anti-malarial drug with less antibacterial activity (MICs $\geq$ 625 μg/mL against *E. coli*).[23] Acetylcholine, a neurotransmitter, has been used as a permeability-active unit in antibacterial agents.[3] Melamine is a nitrogen-rich base, usually chlorinated as antibacterial agents.[24] These amine molecules can anchor onto the surface of gold NPs via Au−N interaction.[25] The physicochemical properties of NPs are shown in Figures S1−S3 and Table S1.

Antibacterial activities of NPs (Au_DAPT, Au_D/NAA, and Au_NAA) and molecules alone are indicated by the minimal inhibitory concentration (MIC) tested via a microbroth dilution method[26] (Table 1). Au_DAPT NPs were active against Gram-negative bacteria but inactive against Gram-positive bacteria. NAA molecules show synergy with DAPT when they were

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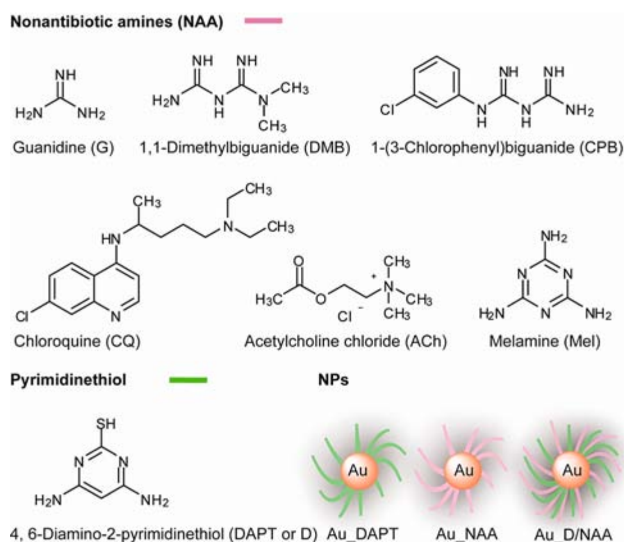

Figure 1. Schematic structures of non-antibiotic amines (NAA) and antibiotics prodrug DAPT used to modify gold NPs individually or in a mixed fashion.

presented as co-ligands on gold NPs (Au_D/NAA). The synergy not only extended the antibacterial spectrum to Gram-positive bacteria but also improved activities against Gram-negative bacteria by 2- to 4-fold. Among NAA molecules, DMB had the most pronounced synergistic effect with DAPT on gold NPs. Au_D/DMB showed MICs of 1 μg/mL against *E. coli*, 4 μg/mL against *P. aeruginosa*, 2 μg/mL against *K. pneumoniae*, and 8 μg/mL against *S. aureus*.

We also tested antibacterial activities of NPs against clinical MDR isolates. MDR Gram-negative strains, *E. coli* BJ915, *P. aeruginosa* BJ915 (these two strains carry extended-spectrum β-lactamase (ESBL) resistance genes, Figure S4), and *K. pneumoniae* R12K2637, were resistant to more than 20 antibiotics and just susceptible to amikacin and colistin (Tables S2 and S3). MDR *A. baumannii* LZ22 was resistant to 13 antibiotics (Table S3). MDR Gram-positive strains, MRSA, *S. suis* SY376, and *E. faecium* JN133, were resistant to more than 12 antibiotics (Table S4). Three classes of antibiotics were used for comparison in Table 1, which are gentamicin (a type of aminoglycosides), mainly used to treat Gram-negative bacteria, levofloxacin (a type of quinolones) with broad spectra, and colisin (a type of polymyxins), used to treat superbugs. MDR strains were all resistant to gentamicin and levofloxacin except MDR *A. baumannii*. Colistin was active against MDR Gram-negative bacteria but naturally inactive against Gram-positive bacteria. In comparison, Au_D/NAA effectively inhibited all tested MDR bacteria regardless of bacterial resistance. Thus, our strategy using DAPT and NAA as co-ligands on gold NPs can effectively deter the resistance of bacteria with broad spectra.

DAPT by itself and each NAA molecule alone had no antibiotic activities against both Gram-negative and Gram-positive bacteria (MICs ≥ 128 μg/mL). Gold NPs individually capped with DAPT, guanidine and its derivatives showed activities only against some Gram-negative bacteria but inactive against Gram-positive bacteria (MICs > 64 μg/mL). CQ-, ACh-, or Mel-capped gold NPs showed no antibiotic activities. Mixtures of Au_NAA (0.5 MIC) or NAAs (256 μg/mL) with Au_DAPT cannot acquire improved efficacy (the same MIC as Au_DAPT, 4 μg/mL against *E. coli* and >64 μg/mL against *S. aureus*). By overnight adsorption of DMB on pre-synthesized Au_DAPT (removing excess DMB molecules via ultrafiltration), NPs show slightly enhanced activity against *E. coli* (MIC 2 μg/mL) but cannot inhibit *S. aureus* (MIC > 64 μg/mL). Hence, the antibacterial activity of Au_DAPT cannot be improved or

Table 1. Antibacterial/Bactericidal Activities Indicated with MIC (μg/mL)/MBC (μg/mL)

		Au_D/NAA NPs						antibiotics [a]			Au_NAA NPs				DAPT or NAA
	Au_DAPT	G	**DMB**	CPB	CQ	ACh	Mel	Gen	Lev	Col	G	DMB	CPB	CQ,ACh,Mel	
Gram-Negative Bacteria															
Laboratory antibiotic- sensitive strains															
E. coli	4/16	2/2	**1**/2	2/2	2/4	2/2	2/2	1/1	<0.25/0.5	0.5/4	16/>64	16/>64	8/>64	>64/>64	≥128/>128
P. a	16/32	16/16	**8**/16	16/16	8/16	16/32	8/16	2/16	2/1	2/8	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128
K. p	2/8	4/8	**2**/8	2/8	2/8	2/8	2/8	1/1	<0.25/0.5	0.5/8	16/32	>64/>64	16/32	>64/>64	≥128/>128
Clinical MDR isolates															
MDR *E. coli* [b]	4/32	2/2	**2**/4	4/4	2/2	2/2	2/2	>64/>64	32/32	2/4	16/>64	>64/>64	16/>64	>64/>64	>128/>128
MDR *P. a* [b]	16/32	8/16	**4**/16	8/16	4/32	4/16	8/16	>64/>64	32/32	2/8	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128
MDR *K. p*	4/64	4/4	**2**/2	4/4	4/4	4/4	2/2	>64/>64	32/32	0.5/0.5	32/>64	>64/>64	32/>64	>64/>64	>128/>128
MDR *A. b*	2/8	2/4	**1**/4	2/4	2/4	2/4	1/4	1/8	0.5/0.5	0.5/4	64/>64	>64/>64	32/>64	>64/>64	>128/>128
Gram-Positive Bacteria															
Laboratory antibiotic-sensitive strains															
S. aureus	>64/>64	16/64	**8**/64	16/64	8/64	16/64	8/64	2/8	0.25/1	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	≥128/>128
S. e	>64/>64	32/32	**16**/64	32/32	16/32	32/64	16/64	1/1	0.25/0.5	64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128
Clinical MDR isolates															
MRSA	>64/>64	32/64	**16**/64	32/64	16/64	32/64	16/32	>64/>64	64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128
MDR *S. suis*	64/>64	16/>64	**16**/>64	16/>64	16/64	16/64	16/64	>64/64	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128
MDR *E. f*	64/>64	16/64	**16**/64	32/64	32/64	32/64	16/64	>64/64	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>64/>64	>128/>128

[a] More data on antibiotic-sensitive activity listed in Table S2–S4. [b] Resistance genes identified by PCR, Figure S4. Antibiotics: gentamicin, levofloxacin, colistin. Strains: *E. coli* ATCC 11775, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, MDR *E. coli* BJ915, MDR *P. aeruginosa* BJ915, MDR *K. pneumoniae* R12K2637, MDR *A. baumannii* LZ22, *S. aureus* ATCC 6538P, *S. epidermidis* ATCC 12228, MDR *S. aureus* MRSA, MDR *S. suis* SY376, MDR *E. faecium* JN133. MIC is the minimal concentration at which no visible bacterial growth is observed in all three parallels. MBC is the minimal concentration at which less than five colonies grow after the subculture on the agar plate post MIC tests.

extended to Gram-positive bacteria by (i) mixing with NAA molecules, (ii) mixing with Au_NAA NPs, or (iii) adsorbing NAA molecules via long-time incubation when Au_DAPT was already synthesized. Synergistic effects occurred only when both of DAPT and NAA were presented as co-ligands on gold NPs during the reduction of $HAuCl_4$.

We tested the bactericidal activities of NPs, antibiotics, and molecules according to the guideline for bactericidal tests,[27] which are indicated with the minimal bactericidal concentration (MBC) following the MIC (Table 1). MBC is the minimal concentration at which less than five colonies grow after the subculture on the agar plate post MIC tests. A ratio of MBC to MIC no more than 4 indicates the bactericidal activity. Au_DAPT showed bactericidal activities against tested most Gram-negative strains. Compared with Au_DAPT, Au_D/NAA showed higher bactericidal activities. By contrast, Au_NAA hardly show any effective bactericidal activity. This assay also proves the synergistic effects of NAA with DAPT on gold NPs on killing bacteria.

We visualized the action of gold NPs (20 μg/mL, the reason for choosing this concentration in SI)) on representatively Gram-positive *S. aureus* by transmission electron microscopy (TEM). Here we visualized that Au_DAPT adhered to the cell wall of *S. aureus* but cannot compromise it (Figure 2A). By

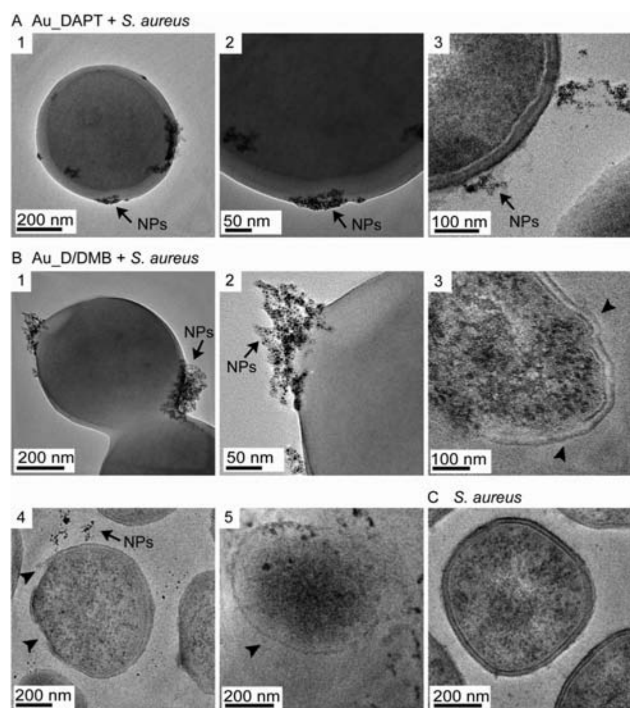

Figure 2. TEM images of *S. aureus* treated with 20 μg/mL of Au_DAPT (A), Au_D/DMB (B), and untreated (C). Arrows indicate NPs and arrowheads indicate compromised cell walls in several types: deformed (B3), broken (B4), and absent (B5). Samples underwent fixing, dehydrating (A1, A2, B1, B2), further cutting superthin slices and staining with heavy metal ions (A3, B3–B5, C).

contrast, representative NPs, Au_D/DMB not only adhered to the bacterial surface but also compromised the cell wall to induce the death of bacteria (Figure 2B). There are several types of compromised structures of the cell wall after Au_D/DMB treatment: loose and deformed (Figure 2B3), lysis and broken (Figure 2B4), and completely absent (Figure 2B5). The normal

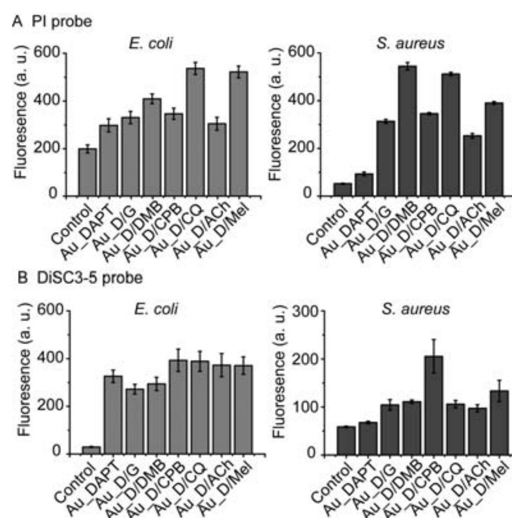

Figure 3. Permeability of bacterial cell membrane probed by PI (A) and diSC3-5 (B). The increase of fluorescence corresponds with the degree of the compromise of total cell membrane (A) and cytoplasmic membrane (B).

appearance of *S. aureus* should be round with the well-organized cell wall (Figure 2C).

We quantified synergistic effects of Au_D/NAA NPs on cell membrane by using fluorescent dyes (Figure 3). Propidium iodide (PI) can cross broken cell membrane and bind to nucleic acids to show enhanced fluorescence.[1] The cyanine dye diSC3-5 (3,3′-dipropylthiadicarbocyanine iodide) is self-quenched inside the organized cytoplasmic membrane. The permeable cytoplasmic membrane can lead to the release of the dye and the recovery of fluorescence.[9a,13,28] Hence, we can use PI and diSC3-5 to assay for the integrity of total cell membrane and cytoplasmic membrane.

For both dyes, increased fluorescent signals indicate compromised membranes. Compared with Au_DAPT, Au_D/NAA can significantly increase cell membrane permeability, particularly when the NAA is DMB, CQ, or Mel (Figure 3A). Au_D/NAA also compromised cytoplasmic membrane of both Gram-negative *E. coli* and Gram-positive *S. aureus* (Figure 3B). Au_DAPT acted weakly on the cell wall of *S. aureus*. These results suggest that Au_D/NAA NPs show wide-spectrum activities by the increase in the permeability of both cell wall and cytoplasmic membranes.

Cell membrane permeable activities suggest the possible anti-biofilm action.[4,8] We found that the synergy of NAA and DAPT on gold NPs can kill bacterial cells in a mature biofilm. Biofilms increase antibiotic resistance and medical device-associated infections.[29] Non-fluorescent fluorescein diacetate (FDA) can be turned into highly fluorescent fluorescein by non-specific intra- and extracellular esterases, which can be used to determine cell viability and biofilm biomass.[30] After treating mature biofilms with NPs for 24 h, Au_D/NAA can significantly decrease the viability of bacterial cells in mature biofilm, whereas Au_DAPT hardly affect it (Figure 4; statistical analysis in Figure S5). In particular, Au_D/CPB eradicated over 95% biofilm cells for both *P. aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive). These results suggest that the synergy of non-antibiotic amines with DAPT on gold NPs can act on biofilm cells.

Synergy of non-antibiotic amines with pyrimidinethiol on gold NPs showed enhanced antibacterial activities by penetrating bacterial cell membrane, which could increase the risk to

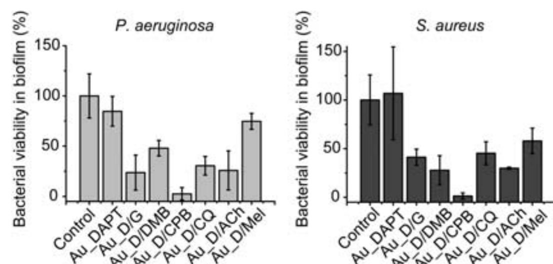

Figure 4. Bacterial viability in mature biofilms tested with FDA. The mature biofilm was exposed to NPs dispersed in nutrient broth medium for 24 h and the bacterial activity was probed with FDA. The assay without NPs is a control.

mammalian cytotoxicity. We tested the cytotoxicity of Au_D/NAA to human umbilical vein endothelial cells (HUVECs) using a commercial CCK-8 kit. These NPs were basically non-toxic to cells under the concentration of 20 μg/mL (Figure S6). The cell viability decreased with the increase of concentrations of NPs. The ratio of half-lethal concentration (LC_{50}) to MIC can reach the maximum of 58 for Au_D/DMB in the case of *E. coli*. The selective activity of Au_D/NAA between bacterial and mammalian cells can be potentially used in the clinical treatment.

In conclusion, we report that the synergistic effects of non-antibiotic drugs and pyrimidinethiol simultaneously presented on gold NPs generated broad-spectrum antibacterial and bactericidal activities against superbugs that resist most antibiotics. These molecules alone show no activity at all. Our study identified an anti-hyperglycemic drug, metformin to have the best synergy with DAPT on gold NPs against both Gram-negative and Gram-positive bacteria including clinical hard-to-treat "ESKAPE" superbugs. This work should broaden antibiotic screening space and help us fight against superbugs. Such strategies may also find uses in other areas of clinical treatments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and strains, synthesis of NPs, antibacterial and bactericidal activity, anti-biofilm, membrane permeability, resistance gene, and antibiotics sensitivity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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