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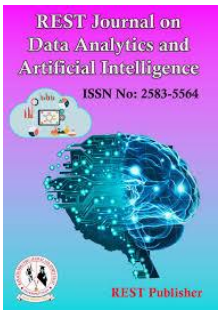

Enhancing Antibacterial Activity Against Staphylococcus aureus with Limonene: A Phytomolecule Potentiator

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Abstract: *Antimicrobial Resistance (AMR) poses a significant threat to global public health - resulting in millions of death every year. With the rampant misuse of antibiotics, it is estimated that the number of cases that succumb to AMR will only rise. Staphylococcus aureus being one of the major 6 major resistant pathogens is known for developing resistance to antibiotics by various mechanisms - one being the extrusion of antibiotics like ciprofloxacin out of the cell through efflux pumps. Efforts to counter this resistance mechanism in S. aureus include the search for efflux pump inhibitors (EPIs). Natural compounds such as limonene, a phytomolecule found in citrus fruits, show promise as EPIs by potentiating the activity of the antibiotic. This paper aims to determine the optimal combination of ciprofloxacin and limonene concentrations required to improve the susceptibility of S. aureus to ciprofloxacin.*

Keywords: *Antimicrobial Resistance, Staphylococcus aureus, Efflux pumps, Ciprofloxacin, Limonene.*

1. INTRODUCTION

Antimicrobial Resistance: Antimicrobial Resistance (AMR) is one of the leading threats to public health in the 21st century. AMR emerges in bacteria as they evolve defense mechanisms against antimicrobial drugs. A paper published in 2022 authored by a cadre of 191 collaborators (self-referenced as Antimicrobial Resistance Collaborators) provided the first comprehensive systematic analysis of global AMR burden. The analysis estimated that in 2019, AMR caused

1.27 million deaths and contributed to 4.95 million deaths worldwide (Antimicrobial Resistance Collaborators, 2022). A previously published review on AMR predicts that by 2050, AMR could be the attributer of 10 million deaths annually. While this projection has been refuted due to the gaps in data about global prevalence of AMR available at the time (de Kraker, M. E et al., 2016), the consensus remains that the incidence of AMR infections is on the rise due to the uncurbed spread of resistant bacterial pathogens (Inoue, H., 2019).

Resistant Bacterial Pathogens and AMR Infections: Of these pathogens, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* take the lead, accounting for over 70% of the total deaths due to AMR. These six bacteria were responsible for 929,000 of 1.2 million deaths attributed to AMR, and 3.57 million of the 4.95 million deaths associated with AMR. Certain pathogen-drug combinations garner more attention, particularly MRSA (Methicillin-resistant *Staphylococcus aureus*), which caused 100,000 deaths in 2019. MDR-TB (Multidrug-Resistant Tuberculosis), 3GC (3rd Generation Cephalosporin)-resistant *Escherichia coli*, Carbapenem-resistant *Acinetobacter baumannii*, Fluoroquinolone-resistant *Escherichia coli*, Carbapenem-resistant *Klebsiella pneumoniae*, and 3GC-resistant *Klebsiella pneumoniae* collectively accounted for 50,000 to 100,000 deaths in the same year (Antimicrobial Resistance Collaborators, 2022). In terms of mortality resulting from infectious syndromes attributed to and associated with antimicrobial-resistant (AMR), lower respiratory tract infections (LRI) exhibit the highest fatality rate, succeeded by bloodstream infections (BSI), intra-abdominal and gastrointestinal infections, urinary tract infections (UTI), tuberculosis, and skin infections. Lower Respiratory Infections (LRI) have the highest death rate - an estimate of 1.5 million deaths in 2019, making it the most burdensome

of the AMR cases globally, especially in LMIC (low and middle income countries) where the means to prevent, diagnose and treat infections are insufficient and sanitation/hygiene is inadequate. (Antimicrobial Resistance Collaborators, 2022) (World Health Organization Fact Sheet, 2020). In the US alone, LRI caused by resistant *Streptococcus pneumoniae* was responsible for about 900,000 cases and 3,600 deaths per year (since 2013) according to the CDC Drug Resistance Threat Report of 2019 (Centre for Disease Control and Prevention, 2019). The overuse and misuse of antibiotics in both human patients and veterinary practices expose bacteria in the environment to antibiotics. Selective pressure and transfer of genes conferring resistance drive the development of antibiotic resistance in bacteria (Riaz, A. et al., 2021). Therefore, in order to combat AMR, it is essential that:

1. The usage and exposure of antibiotics are carefully managed and monitored
2. Adequate preventive and precautionary measures are implemented to curb the spread of AMR
3. Substantial investment is made in researching and developing new antimicrobial drugs or alternative therapies

Mechanisms of resistance in bacteria: Bacteria can acquire resistance to antibiotics through various means, and a specific resistance mechanism isn't limited to just one category of drugs. Different bacteria may employ distinct resistance mechanisms to survive exposure to the same drug. Resistant variants can emerge spontaneously and subsequently become favoured in the presence of the antibiotic. The first category of resistance mechanism is natural - conferred by an intrinsic property of the bacterium. For example, Vancomycin antibiotics cannot penetrate the outer membrane of Gram-negative bacteria, rendering them naturally resistant (Wilhelm, M. P., 1991). Similarly, Mycoplasma, lacking a cell wall, are inherently resistant to antibiotics targeting cell wall synthesis (Gautier-Bouchardon, A. V. 2018). The second category involves acquired resistance due to genetic modifications, including chromosomal and extrachromosomal alterations resulting from spontaneous or induced mutations. These alterations may affect the structure of the target site, reducing the effectiveness or rendering the antibiotic ineffective. For instance, resistant strains of *Staphylococcus aureus* and *Enterococci* develop mutations in genes encoding proteins targeted by antibiotics like Rifampicin (binding to RNA polymerase) and Linezolid (binding to 23S rRNA), leading to structural changes that hinder antibiotic binding (Kakoullis, L., et al., 2021). Horizontal gene transfer, facilitated by elements like R (Resistance) plasmids, transposons, gene cassettes, and integrons, also confers acquired resistance against antibiotics. These genes typically encode enzymes that:

a) Degrade antibiotics: 4 classes of β-lactamases (A, B, C, D) are known to break down β-lactam antibiotics like Penicillins and Cephalosporins (Majiduddin, F. K., et al., 2002).

b) Modify and inactivate antibiotics: Aminoglycosides are inactivated by bacterial acetyltransferases (AACs), nucleotidyltranferases (ANTs), or phosphotransferases (APHs) (Ramirez, M. S., & Tolmasky, M. E., 2010).

c) Modify their own structures for protection: Methylation of ribosomal peptidyl transferase centre (PTC) and peptide exit tunnel (PET) which are target sites for antibiotics like tetracyclines and macrolides (Osterman, I. A., et al., 2020).

d) Actively efflux antibiotics out via efflux pumps: Many heavy metals and other toxic substances including antibiotics like Tigecycline and Tetracycline are actively pumped out of the cell via efflux pumps to decrease their effective concentration within the cell (Kakoullis, L., et al., 2021).

Some antibiotic resistance mechanisms provide cross-resistance, where resistance to one antibiotic extends to others, often those with similar modes of action but sometimes even to entirely different groups of drugs. The accumulation of resistance mechanisms against multiple antibiotics can result in an organism becoming Multidrug-Resistant (MDR) (Hasan, T. H., 2020).

Efflux pumps: Efflux pumps are coded for by highly conserved genes and found in prokaryotes, archaebacteria as well as eukaryotes (Martinez, J. L., et al., 2009). They seem to be evolutionarily ancient systems for protection against not only antibiotics, but also detergents, dyes, antiseptics and heavy metals (Nishino, K. et al., 2021). There are 5 main classes of bacterial efflux pumps which have been identified on the basis of their sequence similarity; which can further be categorised based on their driving energy source:

A] Primary Active Transporters:

1. ABC (ATP Binding Cassette) superfamily

B] Secondary Transporters:

2. Major Facilitator Superfamily (MFS)
3. Resistance-nodulation-division (RND) family
4. Small multidrug resistance (SMR) family
5. Multidrug and Toxic Compound Extrusion (MATE) familyThe Primary Active Transporters (ABC superfamily) are driven by ATP hydrolysis.

Secondary Transporters (MFS, RND, SMR, MATE) are driven by secondary proton gradients/proton motive force. MATE family efflux pumps can also function using the sodium membrane gradient. While ABC, MFS, SMR and MATE efflux pumps are widely distributed in Gram positive and Gram negative bacteria, RND family is exclusive to Gram negative bacteria - forming a tripartite complex spanning across the inner and outer lipid membranes (Piddock, 2006). Apart from expelling harmful substances, various studies have indicated that efflux pumps can participate in intercellular communication by expelling signalling molecules (Martinez, J. L., et al., 2009).

Efflux pumps in *Staphylococcus aureus* and EPIs (Efflux pump inhibitors)*:* *Staphylococcus aureus*, a Gram-positive ESKAPE pathogen, possesses several efflux pumps, primarily belonging to the Major Facilitator Superfamily. Some are linked to resistance against specific antibiotics, such as Tetracycline (TetK, TetL) and Macrolides (MefA, MsrA), while others are multidrug efflux pumps (NorA, NorB, NorC, MepA, and MdeA), which expel both antibiotics and biocides. QacA/B and Smr exclusively expel biocides and are strictly encoded on plasmids, in contrast to the aforementioned efflux pumps, which are encoded on the chromosome, like most efflux pumps (Costa, S. S., et al., 2013a). The chromosomally-encoded NorA MFS family multidrug efflux pump is the most widely studied and well-characterized. Continuously expressed at baseline levels, NorA has the capability to expel hydrophilic fluoroquinolones (ex: norfloxacin and ciprofloxacin), dyes (ex: ethidium bromide), and biocides (ex: quaternary ammonium compounds). Decreased susceptibility to these compounds is attributed to the overexpression of the norA gene (Kaatz, G. W., & Seo, S. M., 1995). NorB exhibits approximately 30% sequence similarity to NorA and imparts resistance against hydrophobic fluoroquinolones (ex: moxifloxacin and sparfloxacin) in addition to the same group of antimicrobial compounds as NorA (Truong-Bolduc, Q. C. et al., 2011). NorC shares about 61% similarity with NorB and is associated with low-level resistance towards hydrophilic and hydrophobic fluoroquinolones and rhodamine dye (Truong-Bolduc, Q. C., et al., 2006). MepA is a MATE family efflux pump which conferrs low-level resistance to quaternary ammonium compounds, ethidium bromide and glycylcycline antibiotics. Fluoroquinolones are weak substrates of MepA (Kaatz, G. W., et al., 2005). Other chromosomally encoded efflux pumps in

S. aureus (MdeA, SepA, SrdM, LmrS) have also been identified. The two main plasmid-encodedefflux pumps are: QacA/B (MFS) and Smr (SMR), both of which extrude out a variety of quaternary ammonium compounds and dyes (Costa, S. S., et al., 2013b). MDR efflux pumps in

S. aureus may contribute to the emergence of multidrug resistance by reducing intracellular drug levels to below therapeutic thresholds. Furthermore, these efflux pumps are found in up to 60% MRSA strains. As a result, there is increasing interest in discovering efflux pump inhibitors (EPIs) (Schindler, B. D., et al., 2013). Research on MDR efflux mechanisms in *S. aureus* has revealed that NorA serves as the primary efflux pump responsible for resistance. Consequently, there is a growing impetus in the search for NorA efflux pump inhibitors (EPIs). Utilizing antibiotic potentiating EPIs could aid in reintroducing antibiotics that have become therapeutically ineffective due to AMR and even in suppressing the emergence of MDR strains (Stavri, M., et al., 2007).

The search for EPIs with improved efficacy begins with phytochemicals. The chemical structures of natural EPIs serve as a good base for further refinements in the search for novel EPIs with increased potency and improved pharmacological profiles. Chalcones, Citral amides, Capsaicin, Piperine, Indole and their derivatives have all been shown to potentiate ciprofloxacin activity in *S. aureus* (Handzlik, J., et al., 2013).

Ciprofloxacin: Ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic, acts by interfering with bacterial DNA supercoiling, a process vital for DNA replication, recombination, and repair. It achieves this by binding to and inhibiting DNA gyrase enzymes. In clinical isolates of *S. aureus*, resistance to ciprofloxacin arises from both mutations

in topoisomerases that impair the drug's binding effectiveness and increased production of endogenous efflux pumps (Shariati, A. et al., 2022). Many synthetic (coumarin derivatives, dihydronaphthalene—imidazole derivatives) as well as natural (indirubin, capsaicin) EPIs have been shown to potentiate ciprofloxacin activity in *S. aureus* (Martin, A. L. A. et al., 2024; Malik, A. A., et al., 2024; Ponnusamy, K., et al., 2010; Kalia, N. P. et al., 2012).

Limonene as an EPI and potentiator of antibiotic activity: Limonene, a monoterpene, is a major component of citrus essential oils (PubChem Compound Database., n.d). While some studies have demonstrated that limonene exhibits antibacterial activity against various bacteria(including *S. aureus*) by compromising the integrity and permeability of the cell wall and interfering with key metabolic pathways such as the Tricarboxylic Acid Cycle (Gupta, A., et al., 2021; Han, Y., et al., 2021), others have suggested contrasting findings (de Araújo, A. C., et al., 2021). Other studies have highlighted the synergistic activity of limonene with antibiotics such as Gentamicin (Sreepian, A., et al., 2022), Ciprofloxacin (Freitas, P. R., et al., 2022), Erythromycin, and Tetracycline (de Araújo, A. C., et al., 2021) against *S. aureus*.

2. AIMS AND OBJECTIVES

1. Determination of MIC (Minimum Inhibitory Concentration) of Ciprofloxacin for S. aureus
2. Determination of MIC (Minimum Inhibitory Concentration) of Limonene for S. aureus
3. Determination of FIC (Fractional Inhibitory Concentration) of Ciprofloxacin and Limonene for S. aureus

3. MATERIALS AND METHODS

Bacterial strains and chemicals: *Staphylococcus aureus* isolate was acquired from the Department of Microbiology at Sophia College (Autonomous), Mumbai. Standard Nutrient Agar/Broth was used for culturing the bacteria. 6M D-Limonene obtained from HiMedia was used for the study. A stock solution of ciprofloxacin was prepared by dissolving a tablet of Ciprofloxacin Hydrochloride (250mg) in sterile distilled water. The antibiotic solution was preserved by freezing. Bacterial cultures were preserved on Nutrient Agar slants at 4°C. The culture was inoculated onto a fresh sterile Nutrient Agar slant and incubated at 37°C for 18-24 hours before use. A standard inoculum was prepared by adjusting the optical density (O.D.) of the saline suspension to 0.8 at 540nm and further diluting it to 1:400.

I. Determination of MIC of Ciprofloxacin for *S. aureus*:

A] A primary antibiotic susceptibility test (AST) was carried out by the agar cup diffusion method. A sterile cork borer (inner diameter = 4mm) was used to bore wells of uniform size into a Nutrient Agar plate swabbed with *S. aureus* culture. Approximately 50µL of Ciprofloxacin at concentrations of 1000µg/mL, 100µg/mL, and 10µg/mL was poured into the agar wells, and the plate was incubated at 37°C for 24 hours.

B] Minimum Inhibitory Concentration (MIC) was determined by the Microbroth dilution method in a sterile 96-well plate containing 200µL of Nutrient Broth with various dilutions of ciprofloxacin (range: 10-100µg/mL at intervals of 10). 5µL of the standard inoculum was inoculated into each well. Positive and negative controls were set up to ensure the validity of the results. Optical density (O.D.) at 540 nm was recorded at 0 hours using an ELISA plate reader. The plate was then incubated at 37°C for 24 hours.

C] MIC was determined by tube dilution method. Dilutions of ciprofloxacin (2µg/mL to 10µg/mL at intervals of 2) were prepared in sterile Nutrient Broth (total volume: 2mL). 50µL of the standard inoculum was inoculated in each tube. Positive and negative controls were set up to ensure validity of the results. All the tubes were incubated at 37°C for 24 hours.

II. Determination of MIC of Limonene for *S.aureus*:

A] A primary antimicrobial susceptibility test (AST) was carried out by agar cup diffusion method. A sterile cork borer (inner diameter = 4mm) was used to bore wells of uniform size into a Nutrient Agar plate swabbed with S. aureus culture. Approximately 50µL of D-Limonene at concentrations of 6M (817.44µg/mL), 10,000µg/mL, 1000µg/mL and 100µg/mL were poured into the agar wells and the plate was incubated in dark at 37°C for 24 hours.

B] MIC was determined by Microbroth dilution method in a sterile 96-well plate containing 200µL of Nutrient Broth containing various dilutions of ciprofloxacin (range: 200-800µg/mL at intervals of 10). 5µL of the standard inoculum was inoculated into each well. Positive and negative controls were set up to ensure validity of the results. O.D. (at 540 nm) was recorded at 0 hour using an ELISA plate reader. The plate was then incubated in dark at 37°C for 24 hours. A viable count was performed after incubation.

C] Determination of MIC by tube dilution method was done in duplicates. 50µL of the standard inoculum was inoculated in 2mL Nutrient Broth with 200µg/mL, 400µg/mL, 600µg/mL and 800µg/mL of limonene. Positive and negative controls were set up to ensure validity of the results. The tubes were incubated in dark at 37°C for 24 hours. A viable count was performed after incubation.

III. Determination of combined effect of Limonene and Ciprofloxacin on *S. aureus*:

A] FIC was determined by the checkerboard method by microbroth dilution in a 96-well plate in duplicate sets. The range of concentration selected for Limonene was 200µg/mL to 800µg/mL at intervals of 200. The range of concentration selected for Ciprofloxacin was 2µg/mL to 10µg/mL at intervals of 2. All dilutions were prepared in sterile Nutrient Broth (Total volume = 2mL). 5µL of the standard inoculum was inoculated in each well. Positive and negative controls were set up to ensure validity of the results. A 0 hour O.D. reading was taken at 540nm using an ELISA plate reader. The plate was incubated in the dark at 37°C for 24 hours.

B] FIC was determined by tube dilution method. The range of concentration selected for Limonene was 400µg/mL to 800µg/mL at intervals of 200. The range of concentration selected for Ciprofloxacin was 6µg/mL to 10µg/mL at intervals of 2. All dilutions were prepared in sterile Nutrient Broth (Total volume = 2mL). 10µL of the standard inoculum was inoculated in each tube. Positive and negative controls were set up to ensure validity of the results. All tubes were incubated at 37°C for 24 hours. A viable count was performed after incubation.

4. RESULTS

I. Determination of MIC of Ciprofloxacin for *S. aureus*:

A] After incubation, zones of inhibition were seen around the agar wells. The diameter of the zone of inhibition for 1000µg/mL, 100µg/mL and 10µg/mL of ciprofloxacin were measured and found to be 28mm, 12mm and 6mm respectively.

B] For the determination of MIC by microbroth dilution, a 24 hour ELISA reading of the plate was taken at 540 nm. 0 hour O.D. values were subtracted from the 24 hour O.D values to eliminate error. The MIC of ciprofloxacin for *S. aureus* was determined to be 8µg/mL.

C] For the determination of MIC by tube dilution, after incubation, all tubes were observed for visual turbidity. 8µg/mL was the lowest concentration of ciprofloxacin at which no turbidity was observed. Hence MIC of ciprofloxacin for *S. aureus* was determined to be 8µg/mL.

II. Determination of MIC of Limonene for *S.aureus*:

A] After incubation, no zones of inhibition were seen around the agar wells.

B] For the determination of MIC by microbroth dilution, a 24 hour ELISA reading of the plate was taken at 540 nm. 0 hour O.D. values were subtracted from the 24 hour O.D.

C] values to eliminate error. The O.D. values were relatively high across all concentrations of Limonene. The viable count was found to be as follows:

TABLE 1.

Tube	Viable Count	% Viability (Relative to Positive Control)	% Inhibition (Relative to Positive Control)
Positive Control	5.33 x 10^(16)		-
Lim 200	8.6 x 10^(16)	161.35	N/A
Lim 400	2.65 x 10^(16)	49.72	50.28
Lim 600	5.05 x 10^(16)	94.75	5.25
Lim 800	1.28 x 10^(17)	240.150	N/A

D] For the determination of MIC by tube dilution, after incubation, all tubes were observed for visual turbidity. All tubes appeared turbid - indicative of bacterial growth. The MIC of Limonene for *S. aureus* was therefore considered to be much higher than what would be clinically significant/useful.

III. Determination of combined effect of Limonene and Ciprofloxacin on *S. aureus*:

A] For the determination of FIC by microbroth dilution, a 24 hour ELISA reading of the plate was taken at 540 nm. 0 hour O.D. values were subtracted from the 24 hour O.D values to eliminate error. Lowest O.D. value (indicative of no/least amount of bacterial growth) at concentrations of Limonene and Ciprofloxacin less than their respective MICs were recorded at 6µg/mL Ciprofloxacin + 400µg/mL Limonene.

B] For the determination of FIC by tube dilution method, 10µL of the contents of each tube was serially diluted, spread plate on sterile Nutrient Agar plates and incubated at 37°C for 24 hours. The viable count was found to be as follows:

TABLE 2.

Tube	Viable Count	% Viability (Relative to Positive Control)	% Inhibition (Relative to Cip 6)
Positive Control	6.21 x 10^(16)	-	-
Cip 6	6.40 x 10^(15)	10.306	-
Cip 6 + Lim 400	3.00 x 10^(14)	00.483	110.951
Cip 6 + Lim 600	3.00 x 10^(14)	00.483	110.951
Cip 6 + Lim 800	5.70 x 10^(15)	09.179	101.256

TABLE 3.

Tube	Viable Count	% Viability (Relative to Positive Control)	% Inhibition (Relative to Cip 8)
Positive Control	6.21 x 10^(16)	-	-
Cip 8	1.92 x 10^(16)	30.900	-
Cip 8 + Lim 400	4.19 x 10^(15)	06.760	134.934
Cip 8 + Lim 600	1.50 x 10^(12)	00.002	144.714
Cip 8 + Lim 800	7.0 x 10^(11)	00.001	144.716

TABLE 4.

Tube	Viable Count	% Viability (Relative to Positive Control)	% Inhibition (Relative to Cip 10)
Positive Control	6.21 x 10^(16)	-	-
Cip 10	2.6 x 10^(12)	00.004	-
Cip 10 + Lim 400	1.8 X 10^(12)	00.003	100.001
Cip 10 + Lim 600	3.5 x 10^(13)	00.057	99.946
Cip 10 + Lim 800	3.72 x 10^(15)	05.990	94.013

5. Discussion:

I] Effect of Ciprofloxacin on S. aureus: The MIC (Minimum Inhibitory Concentration) of ciprofloxacin for S. aureus was determined to be 8µg/mL, showing a total of 30.9% Viability (69.1% Inhibition) as compared to the Positive Control. The MBC (Minimum Bactericidal Concentration - i.e the concentration at which <0.1% cells survive) of ciprofloxacin for S.aureus was determined to be 10µg/mL, showing a total of 0.004% Viability (99.996% Inhibition) as compared to the positive control.

II] Effect of Limonene on S. aureus: Limonene showed no significant inhibitory effect on S.aureus in the selected concentration range of 200-800µg/mL. At low concentration of 200µg/mL and high concentration of 800µg/mL, Limonene seemed have aided in the growth of S. aureus. However, at the intermediate concentrations of 400µg/mL and 600µg/mL, Limonene seemed to show some level of inhibition**.**

III] Combined Effect of Limonene and Ciprofloxacin on S.aureus: Overall, it is observed that limonene (at specific concentrations) works synergistically with ciprofloxacin to decrease bacterial counts. This interaction is more noticeable at lower antibiotic concentrations (sub-therapeutic range, i.e., below MIC) compared to higher antibiotic concentrations. This supports the notion that limonene could be co-administered with antibiotics at lower concentrations, which might not typically be prescribed. This is particularly critical in combating resistance development to higher antibiotic doses while maintaining similar inhibition with lower antibiotic concentrations.

6. CONCLUSION

In conclusion, this study highlights the potential synergistic effect of limonene with ciprofloxacin in combating Staphylococcus aureus, a major contributor to antimicrobial resistance. Limonene could serve as an effective adjunct to antibiotics, enhancing their efficacy at sub-therapeutic levels. This emphasizes importance of exploring alternative therapies to address antimicrobial resistance. Further research is warranted to elucidate the mechanistic aspects and optimize the therapeutic potential of such combinations in clinical settings.

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