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Synergistic Interaction of Glycyrrhizin with Norfloxacin Displays ROS-Induced Bactericidal Activity against Multidrug-Resistant *Staphylococcus aureus*

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Abstract: Acquired bacterial resistance against several antibiotics has severely impaired the drug treatment regime. Multidrug-resistant *Staphylococcus aureus* (MDRSA) causes several life-threatening human pathologies. The introduction of novel antibiotics is a tedious process. Therefore, we have introduced glycyrrhizin (Gly) as a bioenhancer of norfloxacin (Nor), which showed synergistic interactions and a robust drug response. The drug resistance reversal potential of Gly against MDRSA was monitored. Gly and GlyNor (glycyrrhizin + norfloxacin) were used for spectrofluorometer and flow cytometry analysis for the measurement of free radicals and its effect upon cell membranes and macromolecules. Morphological analysis was carried out with the help of SEM. qRT-PCR analysis was conducted for gene regulation. Gly was observed to lower the MIC (minimum inhibitory concentration) of different groups of antibiotics up to 64-fold against MDRSA. GlyNor exerted oxidative stress, as evidenced by the measurement of reactive oxygen species (ROS) and their effect upon cell components. Gly and GlyNor showed membrane damage potential. The expression analysis of oxidative-related and MDR genes showed the up- and downregulation of these genes, respectively. GlyNor significantly lengthened post-antibiotic effects (PAE) and showed reduced mutation frequency rate (MFR). The synergistic bioenhancer properties of Gly with Nor and their enhanced ROS generation against MDRSA are reported for the first time in this study. Severe oxidative stress caused membrane damage, DNA fragmentation, transcriptional changes, and bacterial cell death. We strongly believe this could be a potential measure against rapidly evolving MDRSA.

Keywords: glycyrrhizin; *Staphylococcus aureus*; drug resistance; norfloxacin; oxidative stress

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1. Introduction

The rate of infection of *Staphylococcus aureus* is significantly higher than for other Gram-positive opportunistic bacteria [1]. With the emergence of methicillin (MRSA), vancomycin (VISA/VRSA), and multidrug (MDRSA) resistances, it has become an even greater threat associated with human pathologies [2,3]. It is responsible for the occurrence of various nosocomial (hospital-acquired) and community-acquired diseases [4].

In the past, different natural and synthetic approaches [5] have been exploited to combat this pathogen. Due to the occurrence of acquired resistance in bacteria towards various antibiotics, there is an urgent need to devise ways to control or cure the pathologies associated with drug-resistant strains. It has been reported that several phytomolecules have very little/no antibacterial activity themselves; however, they act as a facilitator (bioenhancer) of these antibiotics [6]. Some phytomolecules such as piperine, lysergol, quercetin, naringin, and niaziridin [7] have been reported to act as bioenhancers and improve the bioavailability of drugs.

One class of phytomolecules, saponins, that belong to glycosides, have many pharmacological activities [8]. Some of them have been reported to be bioenhancers and exhibit membrane-modifying activity [9,10]. For the initial study, we targeted glycyrrhizin (Gly), because this triterpene glycosidic saponin obtained from *Glycyrrhiza glabra* is reported to have bioenhancer properties, for which we obtained an US patent (United States Patent Number US006979471B1-2005) [11]. Traditionally, Gly was used for flavoring and sweetening, although it has been shown that it enhances oxidative stress [12], causes detoxification, and possesses several bioactivities [13,14]. Few studies related to the bioenhancer properties of glycyrrhizin have been reported against other microbial systems [15–18]. This laid the foundation for our hypothesis that glycyrrhizin might show bioenhancer activity against MDRSA. We chose norfloxacin (Nor) for the study because the activity of this fluoroquinolone is rendered inactive because of the recent advance of MDRSA. Another reason is its positive synergistic interaction with Gly.

Oxidants such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) rapidly affect the viability of *S. aureus* cells and cause irreversible damage to the cell membrane and macromolecules, hindering the infection process [19–21]. On the other hand, antioxidants protect the cell from oxidative damage and indirectly enhance the natural defense of the cell [22–24]. Various reports have been published showing the antioxidant potential of phytomolecules [25,26]. Gly is reported to show antioxidant properties that protect cellular components from ROS [27,28]. Farooqui et al. (2018) studied the oxidative stress and dose-dependent cytotoxic effects of Gly in the HPV18+ human cervical cancer HeLa cell line [12]. In the present study, we analyzed whether Gly, along with the clinically used antibiotic Nor, imparts oxidative stress on MDRSA and whether there is a dose-dependent effect. The results revealed that Gly reduced the dose concentration of Nor against the clinical isolates of MDRSA and showed synergistic interactions. We found that Gly and GlyNor enhanced ROS/RNS production and, indeed, caused oxidative stress, leading to bacterial cell death. We also studied whether it influences bacterial membrane properties, as reported in human erythrocytes [29], and observed that it disrupted membrane integrity, compromised membrane fluidity, and affected the membrane potential in bacteria. The MDA content was comparatively higher in GlyNor treated cells and the drug combination showed moderate efflux pump modulatory potential. We observed that GlyNor also caused cytoplasmic leakage in bacterial cells. Additionally, GlyNor caused DNA fragmentation. We also found significant alterations in the expression of the associated genes. Morphologically, the treated cells appeared distorted with deformed architecture. Moreover, GlyNor was found to lower the log CFU/mL value at much lower concentrations than Gly. GlyNor had significantly lengthened post-antibiotic effects and showed a reduced mutation frequency. These results indicate the considerable potential of GlyNor in treating pathologies associated with MDRSA strains.

2. Results and Discussion

The resistance acquired by *S. aureus* towards different groups of antibiotics is one of the main reasons for the increase in the annual morbidity and mortality rate [3,30]. This forces us to introduce novel antibacterial agents against MDRSA on a regular basis [31,32]. This, in turn, requires large sums of money, time, and labor. An alternative to bypass this is the use of phytomolecules, many of which act as bioenhancers or facilitators. The idea behind this is that some phytomolecules have far less antibacterial activity, but have the ability to work synergistically with an ineffective antibiotic (because of the acquired bacterial resistance) to generate a robust drug response [33]. An in vitro combination study was carried out to observe the effect of Gly with four antibiotics (used clinically) against three clinical isolates of *S. aureus* [34]. The MIC (minimum inhibitory concentration) of the antibiotics was found to be reduced 2- to 64-fold in the presence of Gly. Similarly, the MIC of Gly was also found to be reduced 0- to 8-fold with the addition of antibiotics against MDRSA (Table 1). Since Gly was found to interact synergistically with Nor with a lower FICI value of 0.28 against the clinical isolate SA 4627, this combination was used for further study. This activity of

GlyNor has not yet been reported against clinical isolates of MDRSA. Various studies have reported the synergistic interaction of phytomolecules and antibiotics against multidrug-resistant strains [35–40]. However, because of the evolutionary adaptive resistance, there is always a constant need to identify these bioenhancers to improve MDRSA treatment. Therefore, in this study, we identified Gly as a potent bioenhancer that showed synergistic interactions with Nor against the MDR strain SA 4627. We believe that this study reports the bioenhancing activity of GlyNor for the first time.

Table 1. Reduction in the minimum inhibitory concentration (μg/mL) of antibiotics in the presence of glycyrrhizin against clinical isolates of *S. aureus*.

Agent	SA 4627			SA 3721			SA 4753		
	Alone	Combination (Ab/Gly)	Fold Reduction	Alone	Combination (Ab/Gly)	Fold Reduction	Alone	Combination (Ab/Gly)	Fold Reduction
Glycyrrhizin	1600	-	-	800	-	-	800	-	-
Norfloxacin	200	6.25/400	32/4	100	3.12/400	32/2	100	1.56/400	64/2
Oxacillin	800	50/400	16/4	400	50/400	8/2	400	50/400	8/2
Vancomycin	3.12	0.78/800	4/2	0.78	0.04/400	16/2	0.78	0.19/400	4/2
Teicoplanin	3.12	0.78/200	4/8	3.12	0.78/800	4/0	3.12	1.56/400	2/2

Ab = antibiotic; Gly = glycyrrhizin.

Since Gly was able to significantly lower the MIC of antibiotics including Nor, it was targeted for further study as a potential bioenhancer. Following this, we were intrigued to study the reason for this property. We found that GlyNor rapidly induces significant levels of ROS and RNS. In order to evaluate the influence of Gly and GlyNor on the generation of free radicals (H_2O_2 and NO), we performed spectrofluorometric and flowcytometric assays. In the flowcytometry analysis, the non-treated control showed 7.0% DCF-positive cells [34], while at increasing concentrations of Gly, 5.9%, 7.6%, 8.8%, and 11.8% cells were DCF positive. Moreover, 9.6% and 10.5% of the cells [34] were DCF positive for GlyNor and ciprofloxacin at the MIC (Figure 1a). In the case of Gly, we found 9.88 ± 0.31%, 15.58 ± 0.40%, 18.13 ± 0.16%, and 19.68 ± 0.20% H_2O_2 levels at different concentrations, as shown in Figure 1b. GlyNor increased the H_2O_2 level by 24.19 ± 0.31%. Ciprofloxacin, a standard, showed 33.17 ± 0.37% H_2O_2 levels at the MIC (Figure 1b). To investigate the influence of Gly on nitric oxide (NO) generation, the percentage increase in the NO level was calculated. The NO level was increased by 8.5 ± 0.52%, 10.0 ± 1.36%, 17.01 ± 1.86%, and 19.69 ± 3.28% at different concentrations of Gly. At MIC, GlyNor showed an increase in the level of NO of 10.3 ± 0.58%. SNP, a positive control, was found to induce the NO level by 46.33 ± 2.48% (Figure 1c). Our results are in agreement with earlier reports where ROS/RNS generation is induced by some antibiotics and natural products [41]. ROS/RNS causes oxidative stress and therefore exhibits antibacterial properties. It alters the redox homeostasis in the cellular environment and causes irreversible damage to the cellular organelles [42–44]. We also observed that it causes lipid peroxidation, i.e., the oxidative degradation of lipids.

Then, we analyzed the effect of Gly and GlyNor on the bacterial cell wall and cell membrane because oxidative stress also affects the cell membrane [29,45]. No effect on the cell wall was observed alone or with the combination of GlyNor (Figure S1). However, GlyNor exhibited membrane damaging potential and increased lipid peroxidation. Free radicals cause lipid peroxidation, which is responsible for cell membrane destruction that results in modifications to receptors and efflux transporters (required for drug removal) [46,47]. To assess the effect of Gly and GlyNor on membrane damage, a TBARS assay was conducted. The MDA levels were found to be 0.32, 0.37, 0.42, and 0.46 nM at increasing concentrations of Gly, respectively. In the case of GlyNor, the MDA level was found to be 0.326 nM, while the untreated control showed MDA levels up to 0.238 nM (Figure 2a). The efflux pump modulatory/inhibition property of Gly and GlyNor on SA 4627 MDRSA was examined by fluorescence-based ethidium bromide efflux assay. As shown in Figure 2b, in comparison to the control, more fluorescence was identified in the presence of Gly (1.20-fold at 400 μg/mL)

and even more in GlyNor (1.25-fold at 100 + 1.56 µg/mL). These results showed that these compounds have moderate efflux pump modulatory potential. Various reports have been published showing interactions between phytomolecules and antibiotics, inducing efflux pump inhibition/modulation potential [48]. Since this affects the cell membrane, we investigated whether there is any loss of cellular content. We indeed observed the release of cytoplasmic content and extracellular increase in nuclear material concentration. When we applied Gly at the MIC (1600 µg/mL), OD_{260nm} increased 2.4-fold after 60 min of incubation (Figure 2c). However, when we treated the bacterial cells with GlyNor at a much lower concentration (400 + 25 µg/mL), we observed a 4.5-fold increase in OD_{260nm}. These results showed that GlyNor affects membrane properties and causes the release of cytoplasmic content. Cellular damage is characterized by nucleic acid damage [49], apoptosis, and the coagulation of cytoplasmic contents [50].

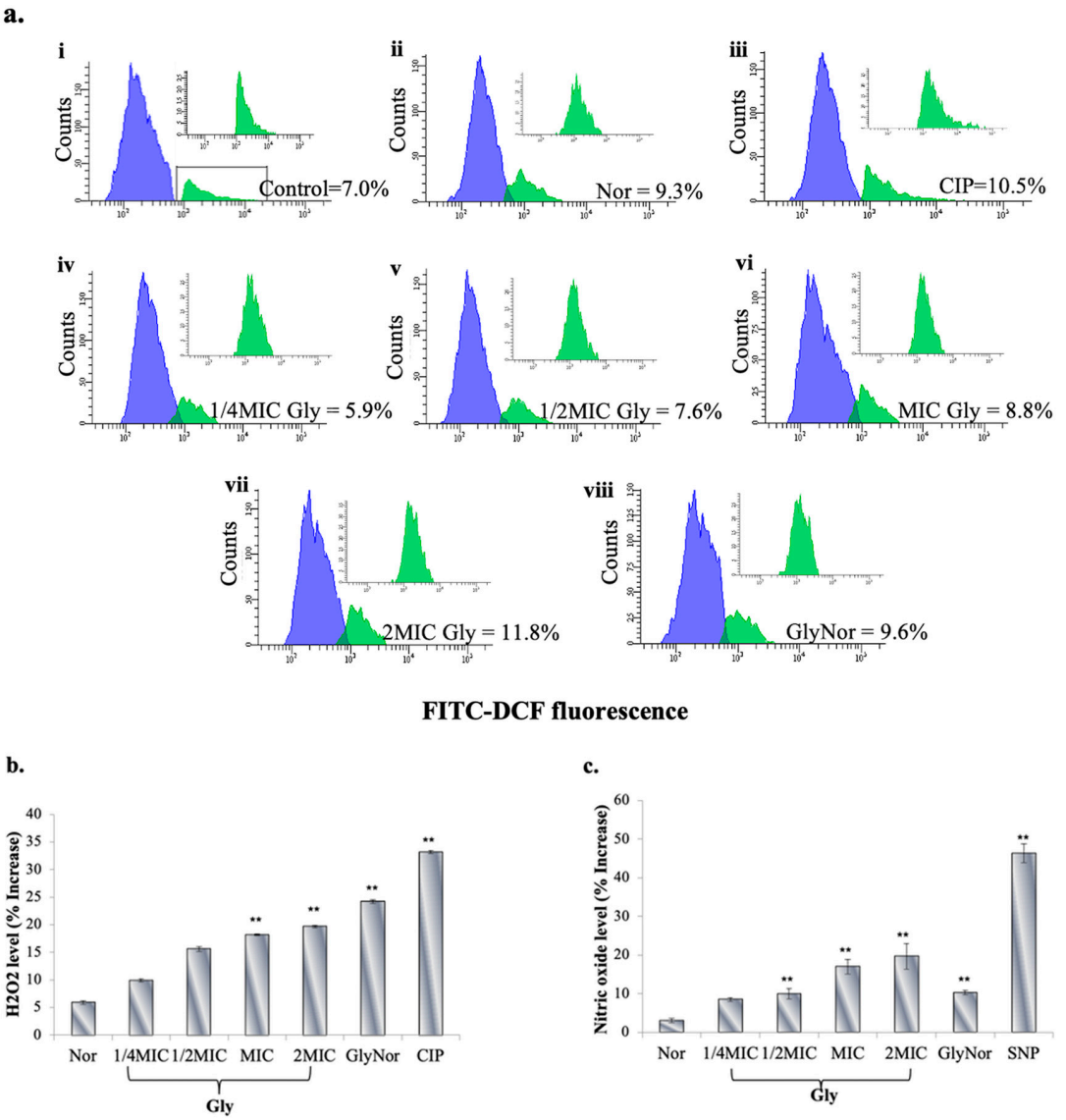

Figure 1. GlyNor caused rapid oxidative stress in SA 4627. (**a**) Flowcytometry histograms showing percentage of DCF-positive cells when exposed to Gly and GlyNor. Data represent two independent experiments with replicated samples. (**b**) Measurement of ROS (H_2O_2) by spectrofluorometry showed generation of free radicals. Data represent two independent experiments with replicated samples. (**c**) Measurement of RNS (NO) by spectrofluorometry showed generation of free radicals. NO measurement was performed in triplicate. Dunnett's test was used to compare the treatment and control, and statistical significance was set at ** $p < 0.01$ vs. control; $p > 0.05$ = not significant.

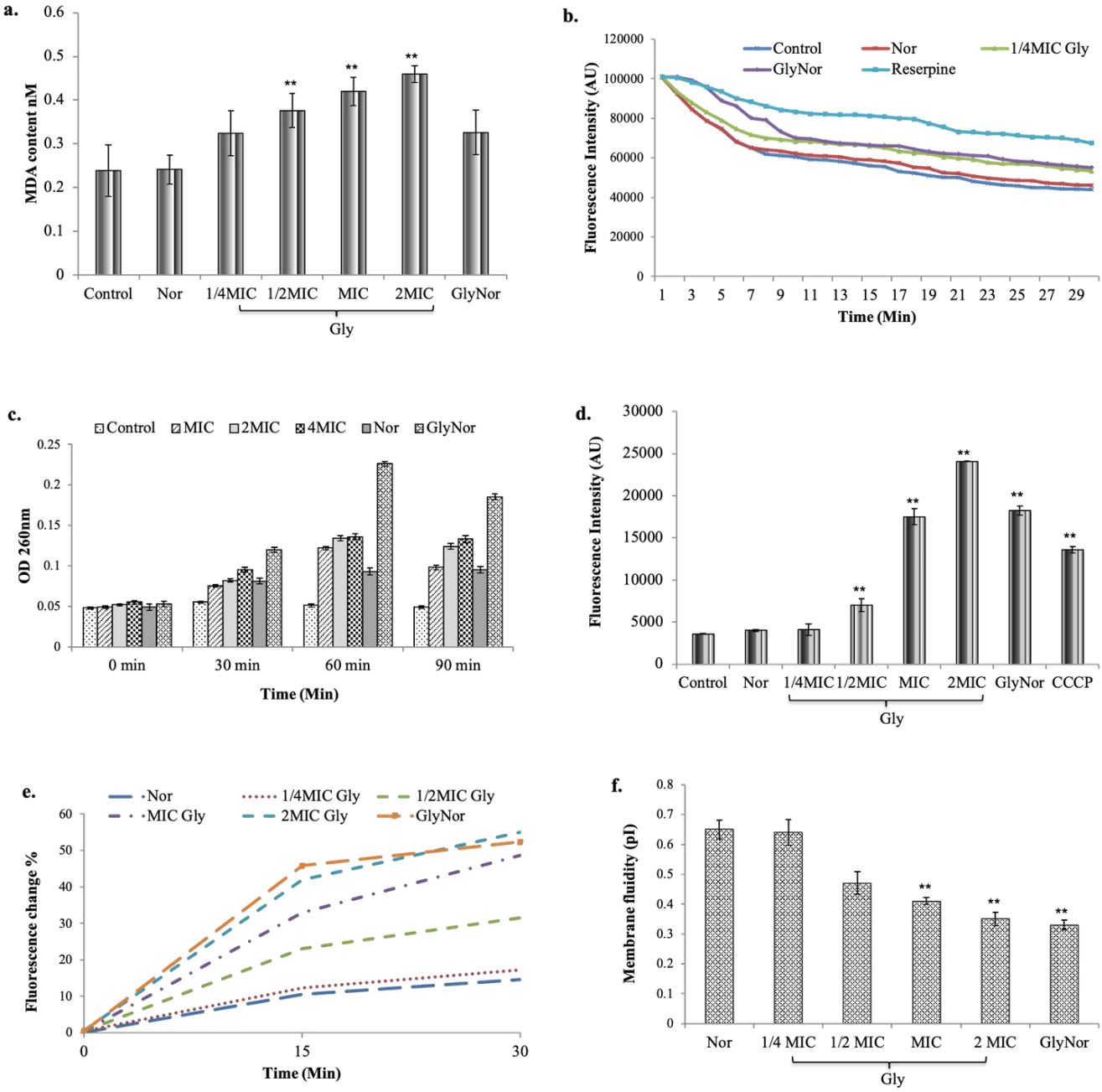

Figure 2. Effect of GlyNor on membrane properties of SA 4627. (**a**) Spectrofluorometric measurement of MDA level showing dose-dependent increase. (**b**) Effect on efflux pump inhibition. (**c**) Effect on cytoplasmic leakage. (**d**) Spectrofluorometric measurement of membrane potential. (**e**) Spectrofluorometric measurement of change in fluorescence representing membrane integrity. (**f**) Fluorescence intensity showing membrane fluidity. The experiments were performed in triplicate. Dunnett's test was used to compare the treatment and control, and statistical significance was set at ** $p < 0.01$ vs. control; $p > 0.05$ = not significant.

The membrane depolarization potential of Gly was analyzed by treating the cells with membrane potential sensitive cyanine dye $DiSC_3(5)$ in the presence or absence of antibiotics. We observed a 4.8-fold increase in the fluorescence intensity after treatment with Gly at MIC (1600 µg/mL) and 5.1-fold after treatment with GlyNor (400 µg/mL + 6.25 µg/mL) (Figure 2d). On further investigation, we found that GlyNor compromises membrane integrity as well as membrane fluidity. These facts demonstrate that GlyNor significantly affects the bacterial cell membrane, which subsequently affects viability [51,52]. In the

case of the membrane integrity assay, *S. aureus* cells treated with Gly (1600 μg/mL) alone exhibited 45.7% fluorescence change, while this was 52.3% in the case of the combined effect of GlyNor (400 + 6.25 μg/mL). This indicated that the combined effect of GlyNor has a notable effect on cell membrane integrity (Figure 2e). Membrane fluidity was evaluated with the help of DPH. A decrease of 26.8% in the fluorescence intensity was observed when the cells were exposed to Gly (1600 μg/mL), while it was 34.1% in the case of combined treatment with GlyNor (400 + 6.25 μg/mL) (Figure 2f). A lower fluorescence value in the exposed cells compared to the untreated control indicates a higher degree of membrane fluidity. These results showed that GlyNor significantly affected the membrane polarization, membrane integrity, and membrane fluidity in the bacteria.

Oxidative stress is reported to severely affect DNA [53,54]. Through a TUNEL assay and a cell viability assay, we observed that GlyNor causes DNA fragmentation and thus eventually affects bacterial viability. A flow cytometer TUNEL assay was carried out to measure the effect of Gly and GlyNor on the DNA. At increasing concentrations of Gly, percentages of TUNEL-positive cells were observed up to 2.8%, 4.7%, 5.4%, and 8.9%, respectively. In the case of GlyNor, the increase was up to 10.3% compared to the untreated control at 2.3% [34]. Ciprofloxacin, a positive control, showed a 9.4% increase in TUNEL-positive cells (Figure 3a) [34]. The transitioning of the bacterial cells towards the FITC green channel and increase in fluorescence intensity indicated that GlyNor induced DNA fragmentation.

This led us to believe that GlyNor might directly or indirectly regulate the expression of associated genes. Therefore, we checked the expression of the various genes involved in ROS and MDR pathways. We understand that transcriptional regulation is a complicated network and many diverse groups of genes are involved in regulatory pathways. For the complete elucidation of these pathways, large-scale genome-wide transcriptomic profiling should be carried out. We performed qRT-PCR to observe the expression of selected oxidative stress and multidrug-resistant genes. *S. aureus* SA 4627 cells were treated with Gly and GlyNor at sub-inhibitory concentrations. We observed that the relative expression of various genes involved in ROS homeostasis rapidly changed in response to induced oxidative stress. Gly- and GlyNor-treated cells showed induced expression in comparison to Nor alone in genes that are associated with antioxidant responses (*sodA*, *katA*, *ahpC*, *tpx1*, and *tpx2*), DNA damage/repair genes (*lexA*, *recA*, and *dps*), iron homeostasis-associated genes (*ftn*, *fur*, and *perR*), and protein damage/repair-related genes (*msrA*, *trxA*, and *trxB*) (Figure 3b). However, within the genes we selected, we found some interesting results. At lower concentrations of ROS, they act as biochemical messengers and help in the activation of various metabolic pathways. However, in higher concentrations, they cause oxidative stress. Therefore, there is an intricate balance between the two that is regulated by a complicated network of various genes. Additionally, bacterial cells protect themselves from oxidative stress by implementing defense mechanisms such as the accumulation of antioxidants and various enzymes [55,56]. At lower concentrations of Gly, the antioxidant activity was at its maximum at 1/4MIC or 1/8MIC (Table S1). These observations could be correlated with the pattern of gene expression wherein at 1/4MIC and 1/8MIC it was found to be upregulated. However, at relatively higher concentrations of Gly and in the case of GlyNor, the antioxidant-related genes showed less expression (ultimately causing oxidative stress). To study the transcriptional profiling of the genes involved in the MDR regulatory pathway, we selected 14 genes. We found that the expression of fluoroquinolone genes (*norA*, *norB*, *norC*, *parC*, *gyrA*, and *sdrM*), the β-lactam group of genes (*pbp2*, *mecA*, and *blaZ*), and genes involved in quaternary ammonium compounds (*qacA*, *qacB*, *qacC*, *mdeA*, and *sepA*) were mostly induced in the GlyNor-treated cells in comparison to Gly or Nor alone (Figure 3c). Transcriptional regulation is a complex mechanism that is regulated at different levels; therefore, other genes might also influence these processes. Similarly, in the case of MDR genes, we found some genes to be downregulated in GlyNor, validating our theory [57,58]. However, some genes showed upregulation, possibly because of the

complicated nature of gene regulation or that they are regulated differently through post-transcriptional, translational, or post-translational modifications.

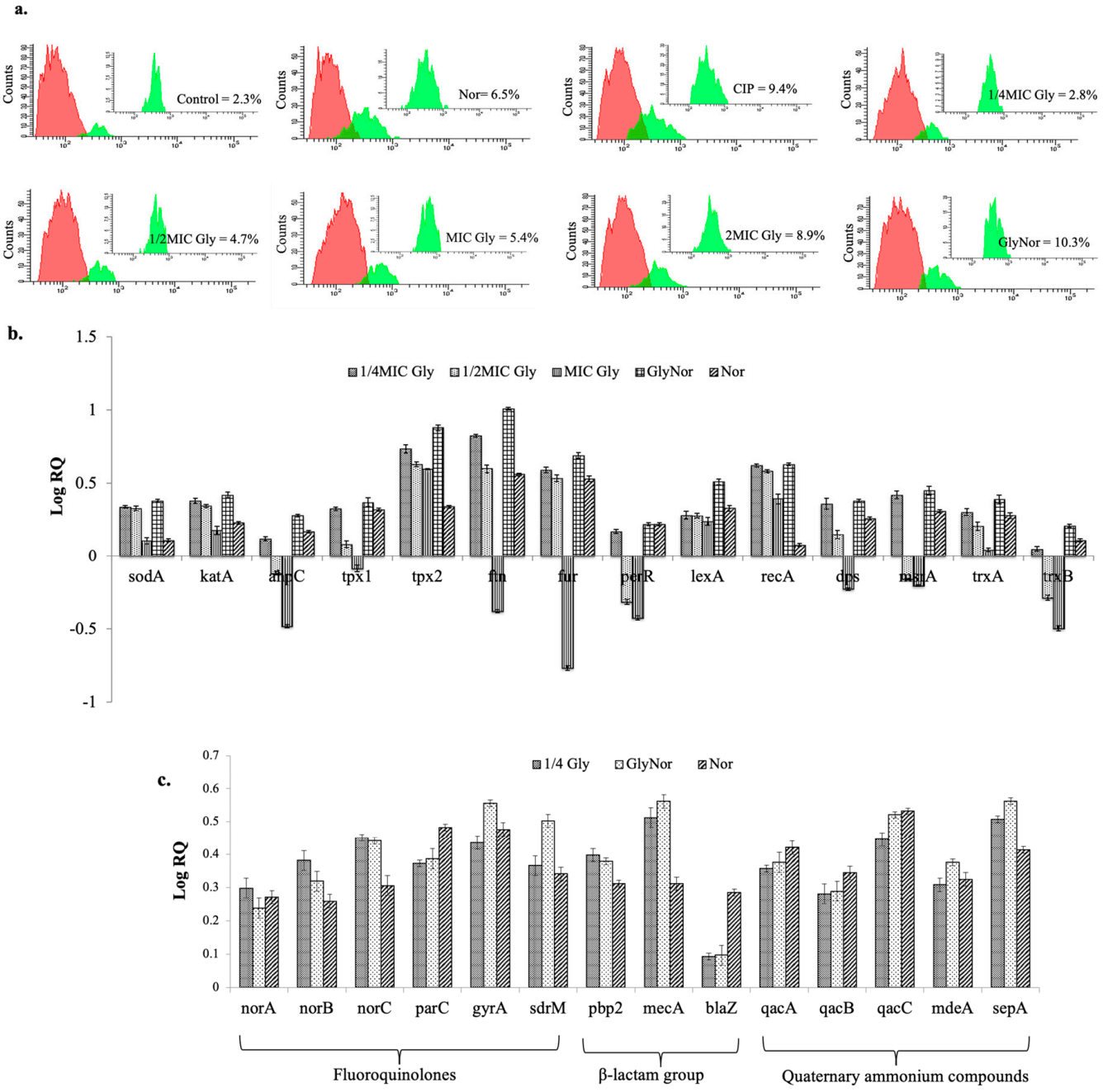

Figure 3. Effect of GlyNor on DNA fragmentation and expression of genes. (**a**) Flowcytometry histograms of TUNEL assay showing the transfer of bacterial population (represented in %) towards green FITC channel. The experiments were conducted in triplicate and only representative figures are shown. (**b**) Gene expression pattern of the genes related to oxidative stress in *S. aureus*. Data are expressed as mean ± SD. (**c**) Gene expression pattern of the genes related to MDR in *S. aureus*. Treatments were conducted with Gly alone and in combination with Nor. Data are expressed as mean values ± SD.

Since the membrane is severely affected, we also checked the overall cell morphology and found that the cells were distorted with a deformed architecture. We observed the cell morphology of MDRSA in the presence of Gly, Nor, and GlyNor. Non-treated control cells were found to be smooth and grape-like; however, not all of them were

spherical. Some were found to be in clusters as well. After treatment with GlyNor at (1/2MIC = 200 + 3.12 μg/mL), the cell morphology appeared to be abnormal. Compared to the non-treated control cells, the treated cells showed deformations with slightly deep craters, possibly because of the membrane deformities and leakage of cellular content. Some cells were found to have blisters on their surface and many cells did not grow to their proper shape/size/length. However, the cells were still recognizable and clustered in large clumps (Figure 4a). These results further support our claim that GlyNor acts as a potent antibacterial agent against MDR. A time-kill kinetic study was carried out to check the cell viability of the Gly- and GlyNor-treated bacterial cells. It was detected that after 24 h of Gly exposure, the log CFU/mL was found to decline from 6.86, 6.86, and 6.81 to 4.11, 3.78, and 3.30 at MIC, 2MIC, and 4MIC, respectively. GlyNor at much lower concentrations could lower the log CFU/mL value from 6.78 to 2.56. The non-treated bacterial cells showed growth from 6.86 to 10.64 log CFU/mL (Figure 4b). For a drug to be effective, it needs certain other criteria as well [59,60]. Therefore, we also monitored the post-antibiotic effect (PAE) of the clinical isolate SA 4627 after treatment with Gly, Nor, and GlyNor for 2 h and found it to be lengthened with the increase in the concentration of GlyNor. We collected samples after every hour thereafter. Over the time period of the experiment, starting from 2 h, the PAE was observed to increase with the increasing concentration of antibacterial agents. The PAE of Nor was increased to 0.8 ± 0.1, 1.4 ± 0.13, and 2.9 ± 0.1 h at different concentrations [34]. The PAE was found to be significantly lengthened to 1.56 ± 0.04, 2.8 ± 0.2, and 3.7 ± 0.14 h at the same concentrations for GlyNor (Gly = 400 μg/mL) (Figure 4c). We also observed that the mutation frequency in GlyNor significantly reduced, which is a desirable trait in any drug. The frequency of mutation prevention concentration is shown in Table 2. No mutant was found or selected from the drug-sensitive strain SA 96. Nor showed a lower mutation frequency compared to Gly, where the growth of the bacterial cells was hampered at 8MIC. In the case of GlyNor at 2MIC, the growth of the bacterial cells was found to be 0.5×10^{-10} CFU. A significant reduction in the resistant mutant strains was found, where the growth was hampered at 100 μg/mL of Gly + 4MIC of Nor (1.56 μg/mL). These findings could be very important for the effective dosing routine of Nor.

We concluded that the bioenhancer Gly interacted synergistically with Nor and exhibited potent bactericidal activity. The probable mechanism of action of GlyNor is that it enhances reactive oxygen species and causes severe oxidative stress. In turn, the oxidative stress generated by GlyNor caused membrane damage and DNA fragmentation leading to bacterial cell death. The expression of several associated genes was altered as well. GlyNor also lengthened the PAE and reduced the mutation frequency rate. Based on these facts, GlyNor has the potential to become an effective drug against MDRSA. We believe the study will provide the benchmark for future research in the area of drug discovery and drug design. This combination study of GlyNor could be extended further to other multidrug-resistant bacteria and parasites, which will pave a new way to combat ever-evolving drug resistance in pathogens. On the other hand, in vivo validation is necessary in these types of drug-design studies, and such studies should be taken up as future research for the further endorsement and recognition of the drug.

Table 2. Mutation frequency of *S. aureus* (SA 96).

Mutation Frequency of *S. aureus* (SA 96) with Nor and Gly					
Agent	**MIC**	**2MIC**	**4MIC**	**8MIC**	**16MIC**
Gly	5.3×10^{-10}	3.9×10^{-10}	2.1×10^{-10}	0.4×10^{-10}	$<10^{-10}$
Nor	3.2×10^{-10}	1.8×10^{-10}	0.6×10^{-10}	$<10^{-10}$	$<10^{-10}$ Ref. [34]
Nor + Gly (100 μg/mL)	2.9×10^{-10}	0.5×10^{-10}	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$

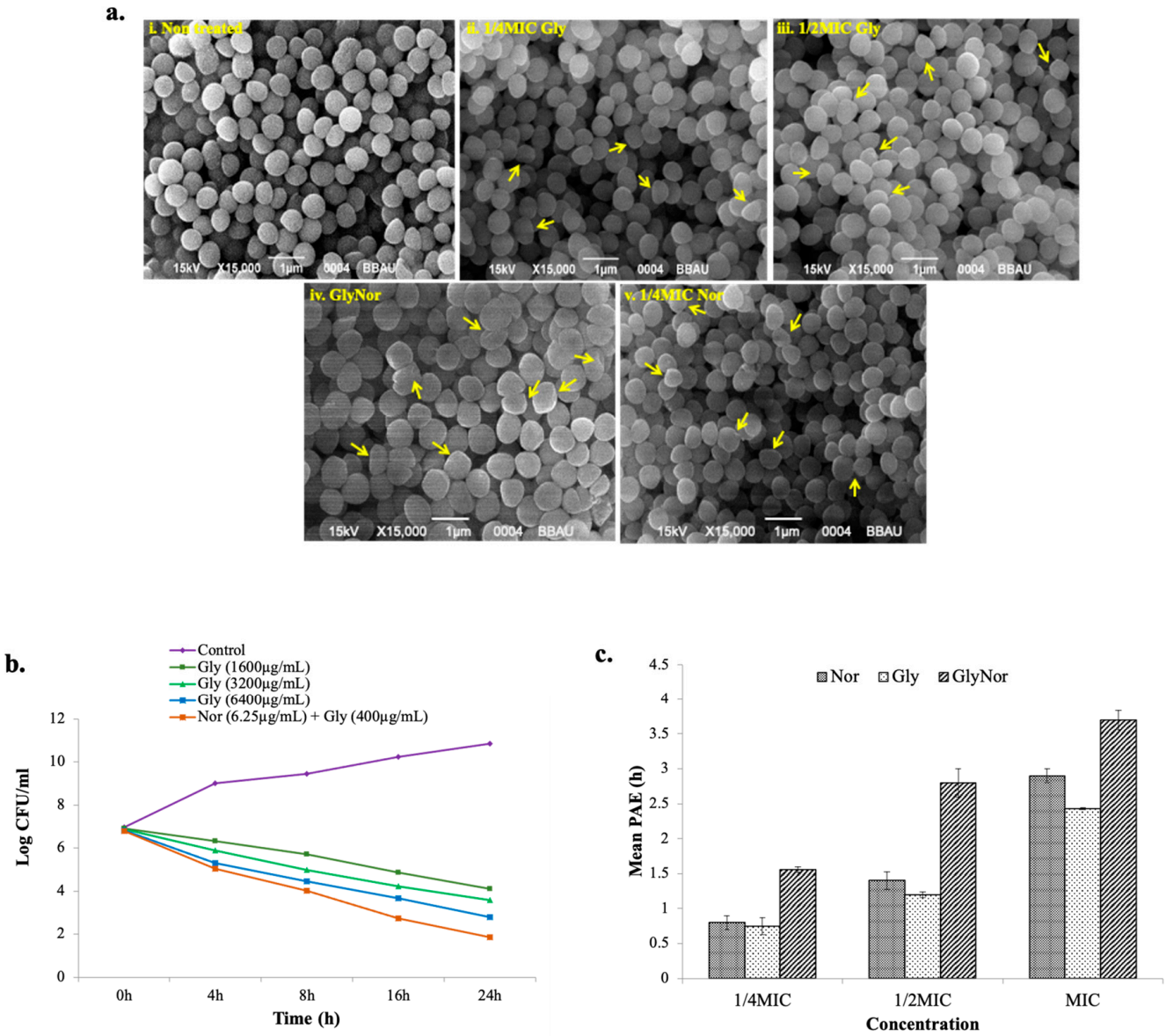

Figure 4. GlyNor affected cell morphology, cell viability, and post-antibiotic effect. (**a**) SEM analysis representing the effect of Gly on cell morphology alone as well as in combination with Nor. A: untreated, B: Gly at 1/4MIC (400 μg/mL), C: Gly at 1/2MIC (800 μg/mL), D: GlyNor at 1/4MIC (100 + 1.56 μg/mL), E: Nor alone at 1/4MIC (50 μg/mL). Yellow arrows were used to indicate the defected bacterial cells. (**b**) Time-kill kinetics of SA 4627 treated with Gly and GlyNor. (**c**) PAE of Nor and GlyNor against SA 4627.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

The drug-sensitive strain (MTCC-SA 96) was obtained from Microbial Type Culture Collection, CSIR Institute of Microbial Technology, Chandigarh, India, and clinical isolates of *S. aureus* were gifted by Dr. K.N. Prasad (Laboratory of Clinical Microbiology, SGPGIMS, Lucknow, India). Standard Mueller–Hinton agar and broth (Hi-Media, Thane, MH, India) were used for culture media. The clinical isolates of *S. aureus* were characterized for their resistance pattern against various antibiotics with different modes of action. The antibiotic sensitivity profiling was carried out using 2-fold serial quantitative (broth dilution) methods [34]. The minimum inhibitory concentrations (MICs) were detected from the observatory data as per CLSI guidelines.

3.2. In Vitro Combination Assay

An in vitro combination study was performed using a broth checkerboard assay [61]. Gly with different groups of antibiotics (Sigma-Aldrich, St. Louis, MO, USA) was used against three clinical isolates: SA 4627, SA 3721, and SA 4753. The two-fold serial dilution of antibiotics was treated with increasing concentrations of Gly. Except for the negative control, each well had a final bacterial inoculum of 5×10^5 CFU/mL and were incubated at 37 °C for 18 h. The fractional inhibitory concentration index (FICI) was determined to test the combination effects of the test compounds. The FICI was calculated by adding the FICs (MIC of drug A in combination with drug B divided by the MIC of drug A alone).

3.3. Measurement of Reactive Oxygen Species

3.3.1. Spectro Fluorimeter Assay

The intracellular ROS was analyzed with the help of CM-H_2DCFDA (Molecular probe, Eugene, OR, USA), a non-fluorescent dye that fluoresces on reaction with ROS in cells, using the method described previously [62]. Briefly, *S. aureus* cultures ($OD_{600\,nm}$ = 0.6) were treated with Gly and GlyNor (Sigma-Aldrich, St. Louis, MO, USA) and washed with PBS twice. CM-H_2DCFDA (10 µM) was applied to the bacterial cells and incubated for 30 min at 37 °C. The cells were obtained by centrifugation and resuspended in PBS (Sigma-Aldrich, USA), followed by mild sonication. The ROS level was determined in the samples by measuring the formation of fluorescent (DCF) using a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). The samples were evaluated at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard.

3.3.2. Flow Cytometry

DCF-positive cells were analyzed with the help of flowcytometry by using CM-H_2DCFDA dye from the method described in Le et al. with minor modifications [63]. The samples were prepared as mentioned. After incubation, the CM-H_2DCFDA-treated samples were observed using an LSRII flow cytometer (BD Biosciences). Unstained cells were examined beforehand for auto-fluorescence in the green emission range. FACSDiva analysis software (BD Biosciences, San Jose, CA, USA) was used to calculate DCF-positive cells and mean fluorescence intensity.

3.4. Nitrite Determination Assay

The nitrite level was quantified using a Griess reagent kit following the manufacturer's protocol with minor modifications [64]. Gly and GlyNor in different concentrations were applied to the *S. aureus* cultures (OD_{600nm} = 0.6). The samples were incubated for 30 min at room temperature and contained 150 µL of culture supernatant, 130 µL of deionized water, and 20 µL of Griess reagent. The nitrite content in the samples was quantified by taking absorbance at 548 nm, using a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). Sodium nitroprusside (SNP) was used as a positive control. The percentage increase in the nitrite production was calculated in comparison to the untreated control. The experiment was performed in triplicate.

3.5. Lipid Peroxidation

A TBARS assay was performed to calculate the MDA level, which is one of the final products of lipid peroxidation in the cells [65]. Bacterial cultures treated with Gly and GlyNor were mixed with 20% (*v*/*v*) trichloroacetic acid. The samples were centrifuged and the supernatant was mixed with 0.8% (*w*/*v*) thiobarbituric acid, followed by incubation at 95 °C for 60 min. The absorbance of the resulting solution was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The level of MDA content was calculated according to the method described [65].

3.6. Ethidium Bromide Efflux Studies

The efflux pump inhibitory/modulator potential of Gly and GlyNor was analyzed using the method described previously [66]. Bacterial cultures were grown up to OD_{600} = 0.6 and then the cells were washed with phosphate-buffered saline. Ethidium bromide was added to the bacterial suspension and incubated for 60 min at 25 °C in the absence/presence of Gly and GlyNor at sub-inhibitory concentrations. Any loss of fluorescence was recorded at the excitation and emission wavelengths of 530 nm and 585 nm, respectively, using a spectrofluorometer (FLUO star omega, BMG Labtech, Ortenberg, Germany).

3.7. Cytoplasmic Leakage Assay

A cytoplasmic leakage assay was carried out to check the effect of Gly and GlyNor on the cell membranes according to Oonmetta-aree et al. (2006) [67]. The bacterial cells were incubated at 37 °C for 18 h. After incubation, the bacterial cells were collected and resuspended in 0.85% NaCl. The cells were incubated with different concentration of Gly and GlyNor. After different time points, the samples were centrifuged and the leakage of low molecular weight metabolites was determined by reading the absorbance at 260 nm using (UV/Vis spectrophotometer Spectramax-190, Molecular Devices, Tampa, FL, USA).

3.8. Membrane Depolarization Assay

ROS generation by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes, and DNA. To observe the effect of ROS on membranes, cytoplasmic membrane depolarization was determined using the membrane potential sensitive cyanine dye $DiSC_3(5)$ (Molecular Probes, Eugene, OR, USA) according to the method described in [68]. In brief, bacterial cells with OD_{600} = 0.6 were collected and washed once with wash buffer (5 mM HEPES, pH 7.2, 5 mM glucose). The cell suspension was incubated with 0.4 µM $DiSC_3(5)$ and 100 mM KCl. The cells were treated with different concentrations of Gly and GlyNor. CCCP was used as a positive control. The fluorescence intensity was monitored by a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

3.9. Membrane Integrity Assay

Membrane permeability was assayed with the LIVE/DEAD BacLight kit (Invitrogen, Waltham, MA, USA) [69]. *S. aureus* cells were grown in MHB media to an OD_{600} of 0.3. The cells were harvested, washed with wash buffer containing 5 mM HEPES and 5 mM glucose (pH 7.2), and treated with different concentrations of Gly and GlyNor. The suspensions were incubated with 5 µM SYTO-9 and 30 µM propidium iodide and incubated at room temperature in the dark. The fluorescence intensity was monitored by a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany) with the excitation wavelength of 485 nm. Emission spectra were monitored at 530 nm (SYTO-9) and 645 nm (propidium iodide). The mean of three independent experiments was considered the result.

3.10. Membrane Fluidity Assay

Bacterial membrane fluidity was observed spectrofluorometrically using 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously [70]. Briefly, bacterial cultures were grown to an OD_{600} of 0.2–0.5 and treated with Gly and GlyNor. The samples were centrifuged, washed with 0.85% NaCl, and resuspended in OD_{600} of 0.3 in 0.85% NaCl + 10 µM DPH. Then, the cell suspension was incubated at 37 °C for one hour. The fluorescence intensity was observed using a spectrofluorometer (FLUOStar Omega, BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 360 nm and an emission wavelength of 426 nm.

3.11. DNA Fragmentation (TUNEL) Assay

DNA fragmentation was measured with the help of an APO-Direct Kit (BD Biosciences, San Jose, CA, USA) [53]. *S. aureus* cultures (OD_{600nm} = 0.6) were treated with Gly and GlyNor at 37 °C. The bacterial cells were washed and resuspended in 4% (*w*/*v*) para-

formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS (pH-7.4) for fixing the cells. The samples were treated with different kits' reagents as per the kit manual and evaluated using flow cytometry (BD Biosciences). FACSDiva analysis software was used to calculate FITC-dUTP-positive cells. Ciprofloxacin was used as a standard. The experiments were run in triplicate.

3.12. Scanning Electron Microscope Assay

The samples were processed for scanning electron microscopy as described previously [71]. Bacterial cells at the mid-exponential growth phase were treated with different concentrations of Gly and GlyNor at 37 °C. The samples were prepared after washing with PBS. Briefly, the samples were fixed with 2.5% glutaraldehyde, followed by dehydration in alcohol and then in 100% acetone. The bacterial suspension was then critical-point dried and examined using a JSM-6490LV scanning electron microscope supplied by JEOL (Japan). Microphotographs were taken at magnifications ranging from X10,000 to X20,000.

3.13. qRT-PCR Analysis

The expression analysis of oxidative stress-related and multidrug-resistant genes were observed by qRT-PCR. Cells were treated with sub-inhibitory concentrations of Gly and GlyNor. Transcriptional profiling was analyzed by the real-time quantification of the RNA templates with the help of SYBR GreenER qPCR super mix (Invitrogen, Waltham, MA, USA) using a 7900HT fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). The relative expression of the genes (LogRQ) in comparison to the untreated control was calculated using the ΔΔct method after normalization with the endogenous control GAPDH [72].

3.14. Time-Kill Studies

The time-kill kinetics of norfloxacin (Sigma-Aldrich, St. Louis, MO, USA) in the presence of Gly and GlyNor were evaluated using the method described previously [73]. Bacterial cultures in the logarithmic phase (1×10^6 CFU/mL) were used for this assay. Different concentrations of Gly (1600 µg/mL, 3200 µg/mL, and 6400 µg/mL) and Nor (12.5 µg/mL) in combination with Gly (400 µg/mL) were tested, respectively. The CFU/mL was observed by a serial dilution method at 0, 4, 8, 16, and 24 h of incubation at 37 °C. Time-kill curves were derived by plotting log10 CFU/mL against time.

3.15. Post-Antibiotic Effect (PAE)

The PAE was determined by the method described in Craig et al. (1991) [59]. A final inoculum of 1×10^6 CFU was incubated with and without Nor, Gly, and GlyNor. After incubation with the antibiotic/s for 2 h, the samples were diluted to 1:1000 to effectively remove the antibacterial agents. Samples were taken every hour until visual turbidity was observed in the control tube. The PAE was evaluated using the formula PAE = T − C, in which 'T' is the time needed for growth in the exposed culture to increase by 1 log10 CFU/mL immediately after drug removal and 'C' is for the corresponding unexposed control.

3.16. Selection of Resistant Mutants In Vitro

The mutation prevention concentration of Nor against *S. aureus* MTCC 96 was calculated using the method described in Heisig et al. (1994) [60]. MHA plates were prepared containing Nor, Gly, and GlyNor. A bacterial suspension of 10^{10} CFU (0.1 mL) was plated. The number of colonies was counted after 48 h of incubation at 37 °C, and the mutation frequency was calculated by the total number of colonies divided by the total number of CFU suspensions plated.

3.17. Statistical Analysis

To analyze the mean values obtained for the treatment and control, first we checked the normality of the data using the Shapiro–Wilk normality test with the rstatix package in

R. After that, we conducted a parametric test, analysis of variance (ANOVA), to analyze significance. Dunnett's test was used to compare the treatment and control, and statistical significance was set at ** $p < 0.01$ vs. control; $p > 0.05$ = not significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ddc2020016/s1, Figure S1: Effect of various concentrations of Gly on cell wall; Table S1: Antioxidant activity of Gly (percent inhibition). Refs. [74–79] are cited in the Supplemenal File.

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Abbreviations

Gly, glycyrrhizin; Nor, norfloxacin; GlyNor, glycyrrhizin in combination with norfloxacin; MDR, multidrug resistance; MDRSA, multidrug-resistant *Staphylococcus aureus*; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; CM-H_2DCFDA, chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate; DiSC3(5), 3,3′-Dipropylthiadicarbocyanine iodide; DCF, dichlorofluorescein; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; SNP, sodium nitroprusside; MDA, malondialdehyde; qRT-PCR, quantitative real-time PCR; SOD, superoxide dismutase; NBT, nitro blue tetrazolium; PBS, phosphate-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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