Synthesis of Calix-Salen Silver Corates for Evaluation of Their Antimicrobial and Anticancer Activities

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ABSTRACT: Silver-based products are becoming popular as antimicrobial agents because of the failure of antibiotics available for tackling the drug-resistant microbial strains. As silver is well tolerated by normal human cells, silver complexes have emerged as important antineoplastic agents. Further, if silver ions are encapsulated within an organic molecule—an azacorand—it may serve as a better substitute for cisplatin or other metal complexes. The calix-salen-type corates were synthesized using silver ions as the template. 5,5'-methylene-bis-salicylaldehyde was reacted with ethylene diamine in methanol at room temperature in the presence of silver nitrate. The resultant corand trapped the silver template in their cavity. The electron-withdrawing and electron-releasing groups like –NO₂, –Br, –C(CH₃)₃, and –OCH₃ were substituted on the bis-aldehyde to study their effects on the antimicrobial and anticaner activities of silver corates. The silver corates were found to have better antimicrobial activity than some of the standard drugs. Bromo-substituted corate-3, nitro-substituted corate-4, and tert-butyl-substituted corate-5 were found to be potent antibacterial agents among all. The bromo-substituted corate-3 was found to be the most potent fungicidal agent among all silver corates. The result of antineoplastic activity suggests that unsubstituted corate-1 and bromo-substituted corate-3 are potential candidates to be used as therapeutic molecules for cancer treatment, which requires further validation.

INTRODUCTION

Supramolecular chemistry has gained the attention of many chemists, biologists, and pharmacists in the last two decades. Supramolecular cavities have been widely used as drug delivery systems, chemosensors, nano reaction chambers, phase-transfer catalysts, and chelating ligands for the extraction of heavy metals from water. One of the various supramolecular entities, the monocyclic cavitand (better known as corand) is capable of encapsulating various metal ions to form metal-corates. The aza-corand binds silver ion preferentially over alkali or alkaline earth metal ions. Silver is known for its medicinal use at low concentration against Gram-negative bacteria, Gram-positive bacteria, and fungi. Silver-based products are becoming popular as antimicrobial agents due to the failure of antibiotics available for tackling the drug-resistant microbial strains. It is proposed that silver ion binds with thiol groups of the cellular proteins of bacterial cells and arrest the normal cellular functions. Silver imparts very less toxicity to humans due to less accumulation in mammalian cells. The main challenge in designing silver-based antimicrobial drugs lies in optimizing slow controlled and sustained release of silver ions over a while. It is well documented that prolonged release of silver ions from their complexes or silver nanoparticles is advantageous over the use of silver salts. The application of silver nanoparticles in the medicinal field suffers from major drawbacks of toxicity to human cells and environmental hazards. Usage of ligands that bind very firmly to the silver(I) center may hinder the timely release of silver(I) ions. The solution to the problem lies in designing supramolecular corands that encapsulate a silver ion in their cavity. The silver aza-corates can be developed as an effective antimicrobial agent with fewer side effects like argyria due to complete encapsulation and minimum exposure of the silver ion.

The clinical success of cisplatin triggered the syntheses of various metal complexes for anticancer activity. The use of cisplatin is also accompanied by many side effects and resistance that cause relapses which makes chemotherapy stressful for the patient suffering from cancer. Various other transition metals like ruthenium, rhodium, iridium, gallium, and gold have been thought as an alternative to platinum and their metal complexes have been studied as antineoplastic agents. Silver being well tolerated by the normal human cells, silver complexes have emerged as important antineoplastic agents. Further, if silver ion is encapsulated within the organic molecule—azacorand—may serve as a better substitute to the cisplatin or other metal complexes.

Despite huge research being done in the area of supramolecular chemistry, the metal corates still have not been well exploited for their medicinal use, except for few calixarenes which have been identified as potent cytotoxic and antitumor
agents.\textsuperscript{12} We synthesized the salen-based corands in methanol using a perchlorate salt of silver ion which acts as the template and also gets bound within the corand. The corands very well stabilize the silver ion in +1 state and prevents autooxidation. The toxicity of Ag\textsuperscript{+} is also thought to get reduced. The silver corates are investigated for their antimicrobial activities and cytotoxic effects.

\section*{RESULT AND DISCUSSION}

Being inspired by the clinical success of silver sulfadiazine, we designed five different calix-salen-type silver corates to study their antibacterial and antifungal activities. We also used these silver corates for the anticancer activity study. The calix-salen-type corates were synthesized using silver ion as the template.

The $S,S'$-methylene-bis-salicylaldehyde was reacted with ethylene diamine in methanol at room temperature in the presence of silver nitrate. The resultant corand trapped the silver template in their cavity. The sodium perchlorate was added to precipitate out the silver corate-1 in the form of its perchlorate salt (Scheme 1). The electron-withdrawing and electron-releasing groups like $-\text{NO}_2$, $-\text{Br}$, $-\text{C}(\text{CH}_3)_3$, and $-\text{OCH}_3$ were substituted on bis-aldehyde to generate corate-2 to -5 in varying yields from 23 to 66\% to study their effect on antimicrobial and anticancer activities (Scheme 1).

The structures of calix-salen silver corates are established based on IR, \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, and 2D NMR measurements including DOSY, HSQC, NOESY, and high-resolution mass spectrometry (HRMS).

The IR spectrum of corate-1 is indicative of its expected structure. $\nu$(OH) is found at 3300 cm$^{-1}$, $\nu$(C=O) is observed at 1632 cm$^{-1}$, and $\nu$(C=N) is observed at 1273 cm$^{-1}$. Comparison of IR spectrum of corand-1\textsuperscript{13} with silver corate-1 shows a shift of $\nu$(C=N) band from 1634 to 1632 cm$^{-1}$, which reveals the binding of silver ion to the azomethine nitrogens of corand-1. Further as sharp medium intensity band is observed at 623 cm$^{-1}$, which confirms the presence of ClO$_4^{-}$ as the counter ion (spectra S-1, S-2, Supporting Information).

\textsuperscript{1}H NMR spectrum of silver corate-1 shows a characteristic singlet at $\delta$ 8.45 corresponding to a proton of imine functionality H$_6$, coordinated to silver ion. The unbound imine proton of the respective corand-1\textsuperscript{13} appeared at $\delta$ 8.37 (spectrum S-3, Supporting Information). The downfield shift of imine proton from $\delta$ 8.37 to $\delta$ 8.34 supports the binding of silver ion with imine nitrogen (spectrum S-4, Supporting Information). Similar behavior was observed by methoxy-, bromo-, and tert-butyl-substituted silver corate-2, -3, and -5 (spectrum S-12, S-17, S-24, Supporting Information). The solubility of nitro-substituted silver corate-4 is comparatively low, hence imine proton is not well resolved (spectrum S-21, Supporting Information). Eight different peaks for a total of 34 carbons were observed in the \textsuperscript{13}C NMR spectra of corate-1 and -3, which propose highly symmetrical structure in the solution state (spectrum S-5, 17, Supporting Information). Corate-2 shows nine different NMR peaks for a total of 38 carbons (spectrum S-13, Supporting Information). NOESY spectrum of corate-1 shows through space interaction between the proton (H$_6$) of the imine linkage with the methylene proton (H$_{9,b}$) of the ethylene group suggesting “E” configuration at the imine bond. The imine proton (H$_6$) also shows correlation with aromatic proton (H$_e$) ortho to imine group. Methylene protons (H$_e$) on the carbon connecting the aromatic rings show correlation with both the aromatic protons (H$_a$, H$_b$) situated at its ortho position, (spectrum S-7, Supporting Information). With the help of HSQC spectrum, all the protons on the respective carbons are assigned precisely (spectrum S-8, Supporting Information). The structure of [2 + 2] cyclocondensed calix-salen-type corate-1 has been confirmed on the basis of the [M + 1] peak observed for corresponding corand at m/z 561.245 in the mass spectrum of silver corate. Here we could not observe the [M + Ag] peak (spectrum S-10, Supporting Information). The possibility of formation of higher macrocyclic corates like [3 + 3] or [4 + 4] along with the [2 + 2] cyclocondensed corate is ruled out on basis of the DOSY spectrum (spectrum S-9, Supporting Information). The spectra of other silver corates 2, 3, 4, and 5 are also in accordance with its expected structure (spectrum S-11 to S-26, Supporting Information). The presence of silver ion was confirmed based on EDS (spectrum S-32, Supporting Information). Cyclic voltammograms confirmed the presence of silver ion in the +1 state (CV-S-27—CV-S-31, Supporting Information).

Previously, we have reported the metal-free synthesis of these calix-salen corands by a high dilution method.\textsuperscript{13} Here we report single-crystal X-ray structure of methoxy-substituted corand. The crystal parameters are listed in the table given below (Table 1). The dimensions of a cavity of the corand are 12.17 Å × 9.98 Å (Figure 1a,b).

The corand molecules are packed to form a two-dimensional rangoli-like array\textsuperscript{14} when observed along the “c” axis (Figure 2). We could not model all the solvent [dimethyl sulfoxide (DMSO)] entrapped in the crystal structure, hence omitted for clarity. Solvent density was found in the extramolecular cavities formed by the crystal packing of the corand leaving the intramolecular cavities empty for encapsulation of other guest molecules.

\textbf{Antimicrobial Activity of Silver Corates.} As some of the organo-silver compounds form prominent group of antimicrobial agents, we decided to study antimicrobial activity of the newly synthesized silver corates and to find out structure–activity relationship, if present. Antibacterial activities of the silvercorates-1 to -5 were tested by treating with different concentrations of the corates and measuring the inhibition of bacterial growth. Three Gram-positive bacterial strains \textit{Staphylococcus aureus}, \textit{Bacillus subtilis}, and \textit{Streptococcus pyogenes} while three Gramnegative bacterial strains \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa}, and \textit{Salmonella typhi} were chosen to carry out the inhibition study. \textit{S. aureus} and \textit{P. aeruginosa} strains are part of the ESKAPE group which are commonly associated with antibiotic resistance.
Ampicillin, chloramphenicol, and ciprofloxacin were used as reference drugs. The minimum inhibitory concentration (MIC) of the corates was determined against these bacterial strains using the broth dilution method. The MIC values of these silver corates are shown in Table 2.

The silver corates show better inhibitory activity against Gram-negative and Gram-positive bacterial strains compared to the standard drug ampicillin (except corate-1 against S. typhi). The most prominent inhibitory activity (MIC = 63 μM/mL) is observed in case of tert-butyl-substituted corate-5 against the Gram-negative bacterial strain E. coli, which is better than those of the standard drugs ampicillin, ciprofloxacin, and chloramphenicol (Table 2). The corate-5 is also potent against Gram-positive bacterial strains S. aureus and S. pyogenes having MIC of 101 μM which is lower than those of the standard drugs ampicillin, ciprofloxacin, and chloramphenicol (Table 2). The inhibitory activity (MIC = 66 μM) against S. typhi and (MIC = 132 μM) against S. aureus of nitro-substituted corate-4 was found to be better than those of all the standard drugs employed in this study (Table 2). The bromo-substituted corate-3 has higher inhibitory activity (MIC = 92 μM/mL) against Gram-negative bacterial strain S. typhi and (MIC = 116 μM) against Gram-positive bacterial strain S. aureus as well as B. subtilis as compared to those of the standard drugs ampicillin, ciprofloxacin, and chloramphenicol (Table 2).

### Structure–Activity Relationship.

It is observed that the unsubstituted silver corate-1 has higher activity against the Gram-positive bacterial strain S. pyogenes with MIC = 130 μM which is lower than those of the standard drugs ampicillin, ciprofloxacin, and chloramphenicol. The substitution on the corate with the lipophilic groups like bromo, nitro, and tert-butyl makes the corate more potent toward the Gram-negative bacterial strains. The reason might be the lipophilicity of the bromo, nitro, and tert-butyl substituents. The cell wall of Gram-negative bacterial strain is highly lipophilic in nature, which will allow and enhance the trespassing of the silver ion when enveloped within the lipophilic carrier. Hence the silver corate-3 and -4 exhibit better activity toward the Gram-negative bacterial strain S. typhi while corate-5 shows the best activity among all against E. coli.

The corates were also screened for their antifungal activity against Candida albicans. The reference drugs used were nystatin and griseofulvin. Minimum fungicidal concentration (MFC) values are reported in Table 3. All corates are found to have better fungicidal activity as compared to the standard drug griseofulvin. Among all the silver corates, bromo-substituted corate-3 has prominent fungicidal activity.

### Cytotoxicity Assay.

Three different cell lines were used to measure cytotoxicity of synthesized corates. Cells were exposed to five different concentrations of corates for 24 h. IC50 value was calculated (Table 4). Among all corates, the unsubstituted corate-1 and bromo-substituted corate-3 showed effective cytotoxicity on IMR 32 cell lines, that is, 5.87 ± 0.91 and 5.1 ± 0.86 μM, respectively. On hepatocarcinoma (HepG2) cell line also, both the compounds showed IC50 value much lower than cisplatin. After corate-1 and -3, methoxy-substituted corate-2 and tert-butyl-substituted corate-5 significantly inhibited the growth of cancerous cells (Figure 3).

### Apoptotic Assay.

Neuroblastoma cells were treated with corate-1 and -3 for 24 h. Poly-I-lysine-coated cells were stained with acridine orange (AO) and ethidium bromide (EtBr) and observed under fluorescence microscope. Necrotic cells were stained with EtBr whereas live cells were stained with AO (green fluorescence, Figure 4).

Cells stained with both stains were apoptotic cells. Treated cells showed a dual staining pattern as apoptotic cells show during cell death. Cells treated with corate-3 showed prominent apoptotic cell death in comparison to those treated with corate-1.

### Table 1. Crystallographic Data for Methoxy Substituted Corand

| Parameter                        | Value     |
|----------------------------------|-----------|
| Formula mass                     | 680.76    |
| Crystal system                   | orthorhombic |
| Crystal size                     | 0.604 × 0.554 × 0.22 |
| Space group                      |Cc        |
| λ (Å)                            | 0.71073   |
| a (Å)                            | 12.7703   |
| b (Å)                            | 24.7400   |
| c (Å)                            | 16.4369   |
| α (deg)                          | 90        |
| β (deg)                          | 90        |
| γ (deg)                          | 8         |
| V (Å³)                           | 5193.1    |
| Temperature (K)                  | 293       |
| Density (g cm⁻³)                 | 1.311     |
| Measured reflections             | 16512     |
| Unique reflections               | 2873      |
| Parameters                       | 171       |
| Restraints                       | 0         |
| Rint                             | 2.33      |
| θ range (deg)                    | 7.598–57.922 |
| R1, wR2                          | 5.67, 17.26 |
| S (Goof)                         | 1.246     |
| Largest diff. peak/hole/(e Å⁻³)  | 0.28/−0.39 |

**Figure 1.** (a) Single-crystal X-ray structure of the corand (b) space fill model of a single-crystal X-ray structure of the corand.

**Figure 2.** A two-dimensional rangoli-like array of the corand observed along “a” axis. Solvent molecules are omitted for clarity.

**Table 2.** The MIC values of silver corates against different Gram-negative and Gram-positive bacterial strains.

| Corate  | MIC (μM) E. coli | MIC (μM) S. aureus | MIC (μM) S. pyogenes | MIC (μM) B. subtilis |
|---------|------------------|--------------------|----------------------|----------------------|
| corate-1| 66                | 130                | 63                   | 92                   |
| corate-2| 104               | 160                | 130                  | 116                  |
| corate-3| 92                | 116                | 86                   | 116                  |
| corate-4| 86                | 116                | 86                   | 116                  |
| corate-5| 58                | 92                 | 58                   | 116                  |

**Table 3.** The MFC values of silver corates against Candida albicans.

| Corate  | MFC (μM) |
|---------|----------|
| corate-1| 63       |
| corate-2| 55       |
| corate-3| 50       |
| corate-4| 50       |
| corate-5| 46       |
Gene Expression Study. The transcript level of different genes was analyzed after treatment with corates. Bax and Bcl2 expression were estimated as they are important markers for mitochondrial-dependent cell death.36 Level of Bcl2 expression was observed to be fold decreased in IMR 32 cells in comparison to the control. Decrease in fold expression of Bcl2 after exposure was 0.1 and −0.9 in corate-1 and corate-3 compounds, respectively. Apoptotic marker Bax transcript level was measured to be fold increased after treatment with corate-1 (1.9-fold increased in expression) and corate-3 (1.98-fold elevated in expression) as compared to control. Tumor suppressor gene, p53 transcript level was also measured and it was expressed as fold elevated which was 2.3- and 2.67-fold increased in expression for corate-1 and corate-3, respectively. Corate-3 showed significant modulations in gene expression in comparison to corate-1 (Figure 5).

### Table 2. Antibacterial Activity of Silver Corates

| corate | E. coli MTCC 443 | P. aeruginosa MTCC 1688 | S. typhi MTCC98 | S. aureus MTCC 96 | S. pyogenus MTCC 442 | B. subtilis MTCC 441 |
|--------|------------------|------------------|---------------|----------------|------------------|------------------|
| 1      | 261              | 261              | 326           | 653            | 130              | 653              |
| 2      | 226              | 113              | 226           | 226            | 282              | 282              |
| 3      | 185              | 231              | 92            | 116            | 231              | 116              |
| 4      | 211              | 211              | 66            | 132            | 211              | 211              |
| 5      | 63               | 101              | 202           | 101            | 101              | 252              |
| ampicillin | 286        | 286              | 716           | 286            | 716              | 716              |
| chloramphenicol  | 155         | 155              | 155           | 155            | 155              | 155              |
| ciprofloxacin | 75           | 75               | 75            | 151            | 151              | 151              |

### Table 3. Antifungal Activity of Silver Corates

| corate | C. albicans MTCC 227 |
|--------|----------------------|
| 1      | 1305                 |
| 2      | 564                  |
| 3      | 462                  |
| 4      | 1057                 |
| 5      | >1009                |
| griseofulvin | 1417       |
| nystatin | 107               |

### Table 4. IC50 (μM) Value of Silver Corates and Cisplatin after 24 h of Exposure

| compounds | L132 IC50 (μM) | HepG2 IC50 (μM) | IMR32 IC50 (μM) |
|-----------|---------------|----------------|-----------------|
| corate-1  | 7.92±0.64     | 7.15±0.88      | 5.87±0.91       |
| corate-2  | 9.34±0.82     | 11.28±1.09     | 7.11±0.86       |
| corate-3  | 8.91±1.2      | 8.39±0.98      | 5.10±0.82       |
| corate-4  | 16.38±1.7     | 22.73±1.3      | 20.61±1.68      |
| corate-5  | 9.59±0.95     | 11.62±1.26     | 9.09±1.08       |
| cisplatin | 26.66±1.8     | 23.66±1.97     | 12.9±1.72       |

Figure 3. Showing 50% inhibition of cell growth on 24 h of treatment of silver corates.

Gene Expression Study. The transcript level of different genes was analyzed after treatment with corates. Bax and Bcl2 expression were estimated as they are important markers for mitochondrial-dependent cell death.36 Level of Bcl2 expression was observed to be fold decreased in IMR 32 cells in comparison to the control. Decrease in fold expression of Bcl2 after exposure was 0.1 and −0.9 in corate-1 and corate-3 compounds, respectively. Apoptotic marker Bax transcript level was measured to be fold increased after treatment with corate-1 (1.9-fold increased in expression) and corate-3 (1.98-fold elevated in expression) as compared to control. Tumor suppressor gene, p53 transcript level was also measured and it was expressed as fold elevated which was 2.3- and 2.67-fold increased in expression for corate-1 and corate-3, respectively. Corate-3 showed significant modulations in gene expression in comparison to corate-1 (Figure 5).

Figure 4. IMR 32 cells stained by acridine orange and EtBr. Untreated cells (B) cells treated with corate-1 and (C) cells treated with corate-3. Dual staining is observed in treated cells (red arrow: apoptotic cell).

Figure 5. Gene expression study on exposure of compound to IMR 32 cells using RT-PCR.

An increased measure of p53 mRNA expression shows the antiproliferative activity of corates. Treatment of cancerous cells with silver corate increased ROS production which changed the mitochondrial membrane potential and escorts the cells for programmed cell death.37,38

### Conclusion
We have synthesized five different silver corates with various substituents like methoxy, bromo, nitro, tert-butyl, and an unsubstituted one using silver ion template. Bromo-substituted corate-3, nitro-substituted corate-4, and tert-butyl-substituted corate-5 were found to be the most potent antibacterial agents among all. Encapsulation of silver in the lipophilic corands ease its entry into the cell through the lipophilic cell membrane of Gram-negative bacteria. It is concluded that the attachment of lipophilic groups on silver corate increases their activity against...
Gram-negative bacterial strains. The bromo-substituted corate-3 was found to be the most potent fungicidal agent among all silver corates. The IC_{50} results indicate that corate-1 and corate-3 are potential candidates for the development of anticancer drugs. Corate-3 showed significant modulations in gene expression in comparison to corate-1.

### METHOD

All the chemicals and reagents were purchased from Sigma-Aldrich and used without further purification. All AR grade or spectroscopic grade solvents were used. Column chromatography was carried out using silica-gel (60–120 mesh). Thin-layer chromatography was performed on precoated silica-gel 60F_{254} (Merck) aluminum sheets. Infrared spectra were recorded on PerkinElmer FT-IR 16PC spectrophotometer. 

H NMR, C NMR, and 2D NMR were recorded on a Bruker 400 MHz NMR spectrophotometer using DMSO-d_{6}. HRMS was recorded from SAIF IIT Chandigarh. Single-crystal X-ray analysis was recorded on a X-CALIBUR EOS Gemini Diffractometer with graphite Mo Ka radiation (0.71073 Å). Melting points were measured in open capillaries and are uncorrected.

5,5'-Methylene-bis-salicylaldehyde and its derivatives were prepared according to the earlier reported procedure.

### General Synthesis of Silver Corate.

Method. (500 mL) placed in a 1 L one-necked round bottom flask equipped with an addition funnel. 5,5'-Methylene-bisaldehyde (3 mmol) and silver nitrate (2.6 mmol, 0.83 equiv) were added to the round bottom flask containing methanol. Ethylene diamine (3.0 mmol, 1 equiv) in methanol (20 mL) was added dropwise from the addition funnel over 1 h to the magnetically stirred solution of bis-aldehyde. Stirring was continued for 2 h. The entire process was carried out in dark. Yellowish turbid solution was obtained. The precipitate was filtered, washed with methanol (4 × 20 mL), and dried under vacuum.

**Corate-1.** Yield: 34.53%. mp range 179−182 °C. IR (KBr disc, cm⁻¹): 3300 (phenol, ν(OH)), 1631 (imine, ν(C=N)), 1629 (imine, ν(C=N)). H NMR (400 MHz, DMSO-d_{6}): δ 13.07 (1H, Hb), 8.45 (1H, Ha), 7.17–6.75 (3H, H_{d,e}), 3.81–3.37 (3H_{f,g}). C NMR (400 MHz, DMSO-d_{6}): δ 167.20 (imine), 159.18; 133.21; 131.89; 131.59; 118.80; 116.31 (aromatic methine), 58.89 (methyl). Mass: 681.296 (M + 1) [Calcd] for corresponding corand.

**Corate-2.** Yield: 36.26%. mp 210 °C. IR (KBr disc, cm⁻¹): 3726 (phenol, ν(O−H)), 1629 (imine, ν(C=N)). H NMR (400 MHz, DMSO-d_{6}): δ 13.74 (1H, H_{d}), 8.35 (1H, H), 7.20 (1H, H_{e}), 6.89 (1H, H_{f}), 3.90–3.86 (3H, H_{g,h}), 1.44 (9H, H). C (DEPT135) NMR (400 MHz, DMSO-d_{6}): δ 167.18 (imine), 130.42; 129.54 (methyl). Mass: 785.492 (M + 1) [Anal.] 785.496 (M + 1) [Calcd] for corresponding corand.

### X-ray Diffraction Studies.

Diffraction data for corand-2 were collected on an X-calibur, EOS, Gemini diffractometer with graphite monochromatic Mo Ka radiation (0.71073 Å). All structures were solved and refined using the Olex2 software and ShelXL refinement package. Graphics were generated using MERCURY, version 3.9. All structures were solved by the direct method. CCDC no: CCDC 1917152.

### Antimicrobial Activity.

Broth dilution method was used to determine MIC values for Gram-positive and Gram-negative bacterial strains and to determine MFC values for *C. albicans*. DMSO was used as the diluent/vehicle to get the desired concentration of the test compounds to measure their activity against standard bacterial strains. Silversulfadiazine and ciprofloxacin were the standard antibacterial drugs used for comparison of MIC values while griseofulvin and nystatin were used as the standard antifungal drugs for comparison of MFC values.

Mueller Hinton broth was used as the nutrient medium to grow and dilute the drug (or compound) suspension for the test bacteria. Inoculum size for test strain was adjusted to 10^8 CFU [colony forming unit] per milliliter by comparing the turbidity. The strains were procured from Institute of Microbial Technology, Chandigarh.

### Cytotoxicity Assay.

In vitro anticancer activity of silver corates was carried out against human cancer cell lines. IMR 32 (neuroblastoma cell line), HepG2 and L132 (immortal lung cell line) were procured from the NCCS, Pune. MTT assay, according to the standard protocol, was performed against these three cells. Cisplatin is one of the effective chemotherapy drugs, which was used as a reference drug in the present study. Each cell line used (IMR32, L132, and HepG2) was exposed to different corate compounds with different concentrations (2, 6, 10, 14, and 18 μM/200 μL) for 24 h. Cells were exposed to silver corate compounds for 24 h. The relationship between compound concentration and cell viability was measured to calculate IC_{50} values, the values that show 50% inhibition of cell viability.

### Gene Expression Study (qRT-PCR).

For analysis of mRNA transcript variation, cells were treated by silver corates for 24 h. RNA was extracted and quantified by Nanodrop spectrophotometer. cDNA was synthesized using Bio-Rad iScript CDNA synthesis kit and used as the template for RT-PCR. The mRNA transcript level of p53, Bax, and Bcl2 genes were studied. β-actin was used as the reference gene. Primers of the target gene were designed using BLAST software of NCBI. The sequence of primers is given here. (p53 primer F:

Gram-negative bacterial strains. The bromo-substituted corate-3 was found to be the most potent fungicidal agent among all silver corates. The IC_{50} results indicate that corate-1 and corate-3 are potential candidates for the development of anticancer drugs. Corate-3 showed significant modulations in gene expression in comparison to corate-1.

**Corate-4.** Yield: 29.10%. mp > 280 °C. IR (KBr disc, cm⁻¹): 1649 (imine, ν(C≡N)), 1535 (nitro, νas(NO{2})), 1350 (nitro, νs(NO{2})). H NMR (400 MHz, DMSO-d_{6}): δ 8.21 (1H, H_{d}), 7.70–7.66 (1H, H_{e}), 7.34–7.17 (1H, H_{f}), 5.56 (1H, H_{g}), 3.86–3.67 (3H, H_{h}). Mass: 740.011 (M') [Anal.] 740.183 (M') [Calcd] for corresponding corand.

**Corate-5.** Yield: 36.26%. mp 210 °C. IR (KBr disc, cm⁻¹): 3726 (phenol, ν(O–H)), 1629 (imine, ν(C=N)). H NMR (400 MHz, DMSO-d_{6}): δ 13.74 (1H, H_{d}), 8.95 (1H, H), 6.89 (1H, H_{e}), 3.90–3.86 (3H, H_{g,h}), 1.44 (9H, H). C (DEPT135) NMR (400 MHz, DMSO-d_{6}): δ 167.18 (imine), 130.42; 129.54 (methyl). Mass: 785.496 (M + 1) [Calcd] for corresponding corand.

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5′CATAGTGTGGTGTCGCCTA3′, R: 5′CACCT-CAAAGCTGTCTCCGC3′; Bcl2 primer F: 5′GCCCGAGAACCCTAATGCTT3′, R: 5′CTCAGGGGACT-CACCTGCT3′; Bax Primer: F: 5′GCCCTTGTGCTTCAAGGCTT3′, R: 5′GGAAGAAGACCTCTTGAGGG3′; and β-actin primer F:5′ CCACC-CTGTACCCTGGCATT3′, R: 5′ CGCTCAGGAGGAGCAATGAT3′). Real Time PCR reactions were performed using Power up SYBR Green mix (invitrogen). Calculation of CO2 incubator. IMR 32 cells were treated with silver corates 32 cells were seeded and incubated overnight for adhesion in a cell death by dual CAATGAT3′ according to the fold di CATGTACCCTGGCATT3′, R: 5′ CGCTCAGGAGGAGCAATGAT3′. Real Time PCR reactions were performed using Power up SYBR Green mix (invitrogen). Calculation of relative gene expression (normalized by β-actin) was analyzed according to the fold difference in expression using the 2−ΔΔCT method.41

Cell Death Analysis. AO and EtBr were used to observe cell death by dual fluorescence staining mechanism. Poly-L-lysine-coated coverslip was kept in 6-well plates on which IMR 32 cells were seeded and incubated overnight for adhesion in a CO2 incubator. IMR 32 cells were treated with silver corates for 24 h. Cells were stained by AO/EtBr (4:1) for 20 min in dark at room temperature. Cells were washed with PBS and observed under a fluorescence microscope.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02948.
IR, NMR, HRMS spectra, cyclic voltammogram, EDS spectrum (PDF)

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Notes
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