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Ecology of supramolecular entities of antibiotics (SMEAs), on-demand controlled guest capture and release systems: tuning spacer and polarities in abiotic and biotic factors

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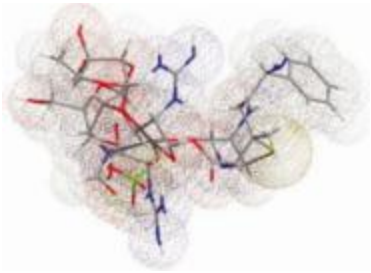
ABSTRACT



Antimicrobial Potential: Screening Area of Supramolecular Entities of Antibiotics (SMEAs)

Earlier reported supramolecular entities of antibiotics (SMEAs) synthesized from various antibiotic drugs were tested to find out their antimicrobial properties i.e. Antibacterial activity against *Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa*), and Antifungal activity against *Candida albicans, Aspergillus flavis and Candida glaberata*), and showed varying degree of percentage inhibition. Antibiotics medications no longer respond to antifungal and antibacterial infections, proposed SMEAs designed to get rid of it. The anti-fungal activity experiments are difficult due to slow growth of fungi and resemblance to host activity. The SMEAs assimilation through molecular mechanisms on microbes was explored through computational models and found suitable as potential antimicrobial agents against different microbes. The assimilation properties are dependent on their good adsorptive ability and that's because of the interactions that occurred between microbial and backbones of SMEAs.

Keywords: Supramolecular Entities of Antibiotics (SMEA), Antibiotic Drugs, Antibacterial activity, Antifungal activity

INTRODUCTION

Infectious diseases are generally caused by microorganisms. They derive their importance from the type and extent of damage their causative agents inflict on organs. Damage to tissues mainly results from the growth and metabolic processes of infectious agents intracellularly or within body fluids, the production and release of toxins or enzymes that interfere with the normal functions of organs.[1,2] These products may yetbe distributed and cause damage in other organs or functions such that when the pathogen consequently invades more organs. Naturally the host's elaborate defence mechanismsand immune system fights the infectious agents and eliminates them. Infectious disease results or emerges in instances when the immune system fails to eliminate pathogenic infectious agents.[3,4] However, some pathogens, after apparent elimination and a period of dormancy, are able to acquire properties that enable them to reinfect their original or new hosts, usually in increasingly alarming proportions. This summarizes the most important antifungal and antibacterial agents and their most common uses.[5-7] For this reason, the development of new antifungal and antibacterial agents, preferably with novel mechanisms of action, is an urgent medical need. Hence, substantial attention has been focused on developing a more detailed understanding of the mechanisms of antimicrobial resistance, improved methods to detect resistance when it occurs, new antimicrobial options for the treatment of infections caused

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by resistant organisms, and methods to prevent the emergence and spread of resistance in the first place.[8]

In addition, numerous secondary metabolites, such as various antibiotic drugs etc. were structures that later became the basis for synthetic and semisynthetic derivatives with improved pharmacological properties. Besides host and environmental factors, changes or mutation in the genome of a pathogen occurs as a result of exposure to chemicals and antimicrobial agents (e.g., antibiotic), may lead to gene damage and emergence of drug resistant pathogen variants that could cause new diseases.[9-10] Increasing resistance of microorganisms against available antimicrobial agents is of major concern among scientists and clinicians worldwide. In general, it was observed that pathogenic viruses, bacteria, fungi, and protozoa are becoming more and more difficult to treat with the existing drugs. To overcome this drawbacks of the current antimicrobial drugs and to obtain more efficacious drugs, an antimicrobial drug having a novel mode of action should be developed.[11]

A major challenge was to measure quantitative differences consistently and accurately in bioavailability between multiple forms of inorganic metals in the studied environment. Thus, the design of smart drug delivery systems has been an area of growing interest. SMEAs showed their host-guest interactions within donor set and wide open their selectivity and functionality. These newer agents displayed significant antibacterial activity against different pathogens like *Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa*. The antifungal activity assessment of these SMEAs was done against different pathogens like *Candida albicans, Aspergillus flavis and Candida glaberata*. Reported SMEAs showed higher antimicrobial activity in comparison to metal-free ligands and may play a role in the development of better anti-microbial agents for biomedical requirements.

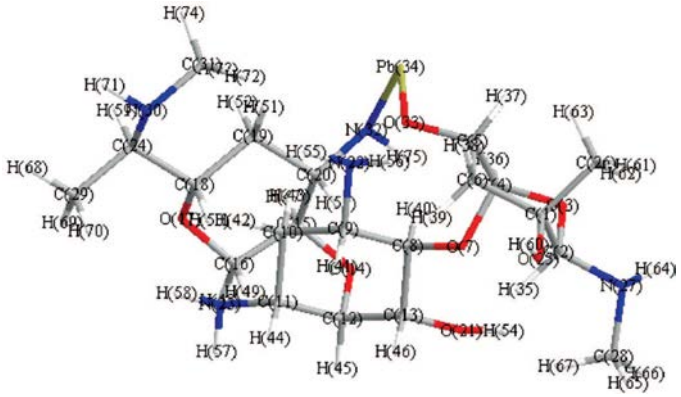
MATERIALS AND METHODS

Supramolecular Entities of Antibiotics (SMEAs)

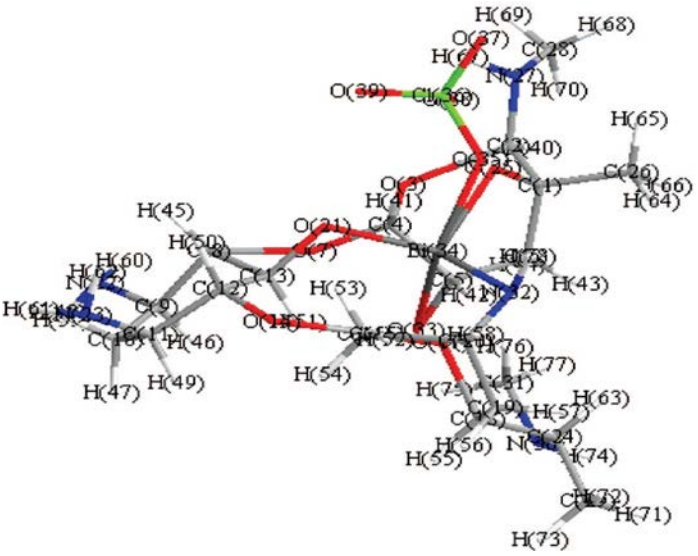
Earlier reported ligand (L-1) and SMEA-1 and 2 of Bi(V) and Pb(II) derived from 2-[4,6-diamino-3-[3-amino-6-(1-methyamino-ethyl) tetrahydropyran -2-yl]oxy-2-hydroxy-cyclohexoxy]-5-methyl-4-methylamino-tetra hydropyran-3,5-diol, ligand (L-2) and SMEA-3 of Bi(V) consist of 5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)-4-[4,5-dihydroxy-6-(hydroxylmethyl)-3-methyl amine-tetrahydrop-ya-2-yl]oxy-3-hydroxy-2-methyl-tetrahydro furan-3-carbaldehyde and (2S,5R,6R)-6-[(8)-2-amino-2-phenylacetamido]-3,3-di-methyl-t-oxo-4-thia-1azabicyclo[3.2.0] heptane-2-carboxylic acid, and ligand (L-3) and SMEA-4 and 5 of Bi(V) derived from amoxicillin trihydrate (ACT) and ampicillin trihydrate (APT), and ligand (L-5) and SMEA-6, 7 and 8 of Mn(II), Co(II), and Ni(II) synthesized from (6R)-6-(α-phenyl-D-glycylamino) penicillanic acid[12-15] reproduced in fig. 1 to 4, were used for antimicrobial screening.

Microbiological Studies : Test Materials

SMEAs were dissolved in ethanol:hexane (1:1) using 1% Tween 80 solution at a final concentration of 512 µg/ml and sterilized by filtration using 0.22 µm Millipore (MA 01730, USA) and used as stock solutions. Ketoconazole, and Imipenem were used as standard antibacterial and antifungal drugs. Reference antibacterial agents were obtained from their respective manufacturers and dissolved as per standard procedure.[16]



SMEA-1



SMEA-2

Figure 1. Stick numeration models of SMEAs. Color code: Pb, yellow; O: red; C, light gray; H, white; N, blue.

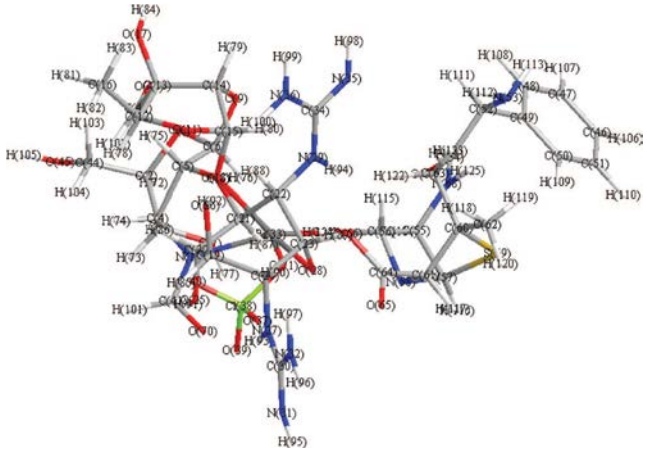
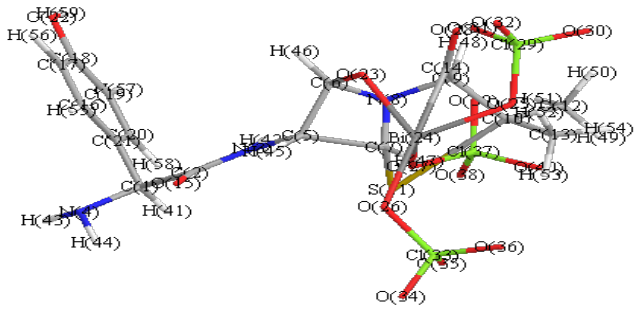
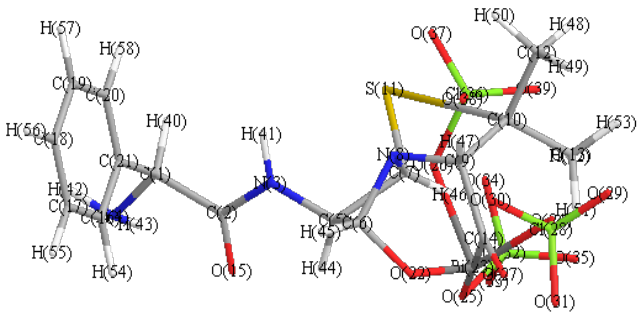


Figure 2. Stick numeration models of SMEA-3. Color code: Bi, dark grey; O: red; C, light grey; H, white; N, blue.



Skeleton of SMEA-4



Skeleton of SMEA-5

Figure 3. Stick numeration models of SMEAs. Color code: Bi, dark grey; O: red; C, light grey; H, white; N, blue.

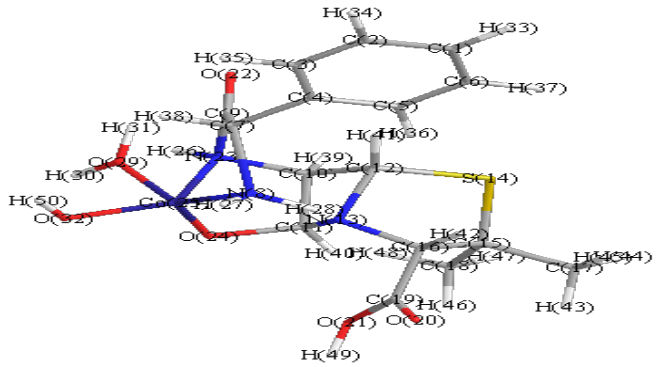


Figure 4. Stick numeration models of SMEA-6 to 8. Color code: Bi, dark grey; O: red; C, light grey; H, white; N, blue. M = Co(II), Ni(II) and Mn(II).

Antibacterial activity tests were carried out against standard and isolated strains.

Culture Media

Mueller Hinton broth (MHB; Difco) and Mueller Hinton agar (MHA; Oxoid) were applied for growing and diluting the bacteria suspensions.[17a]

Inoculum

The microorganism suspensions used for inoculation were prepared at 105 CFU (colony forming unit/ml) by diluting fresh cultures at McFarland 0.5 density (108 CFU/ml). Suspensions of bacteria and fungi were added in each well of the diluted test solutions, density of 105 CFU/ml for fungi and for bacteria. The bacterial suspensions used for inoculation were prepared at 105 CFU/ml by diluting fresh cultures at McFarland 0.5 density (108 CFU/ml). The fungi suspension was prepared by the spectrophotometric method of inoculums.[17b]

Antibacterial and Antifungal Activities

The microdilution method was employed for antibacterial and antifungal activity tests. Media were placed into each 96 wells of the microplates. Test solutions at 512 µg/ml were added into first rows of microplates and two-fold dilutions of SMEAs (256–0.125 µg/ml) were made by dispensing the solutions to the remaining wells. 10 µl culture suspensions were inoculated into all the wells. Ethanol:hexane (1:1) using 1% Tween 80, pure microorganisms and pure media were used as control wells. The test was carried out in triplicate in each run of the experiment. The sealed microplates were incubated at 35°C for 24 hrs and 48 hrs in humid chamber. The lowest concentration of the compounds that completely inhibit macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported as described previously.[17c-d] The MICs were evaluated on test samples that showed antimicrobial activity.

Hanging Drop Method-Antifungal Activity

The concentration of 500 ppm the test compounds was used to study the antimicrobial activities on germination of fungal spores by the hanging drop method. The germination of the spores was observed under microscope after 8 hours of incubation at 30°C for incubation period of 5-8 days. The percentage inhibition of spore germination was calculated using the equation, % Inhibition of spore germination = Total number of germinated spore/ Total number of spore.[18]

Agar Well Diffusion Method-Antibacterial Activity

The antibacterial activity was determined using agar well diffusion method. The wells were dug in the media with a sterile borer and eight-hour bacterial inoculum containing ca. 104-106 colony-forming units (CFU)/ml was spread on surface of nutrient agar using a sterile cotton swab. The recommended concentration of best sample (2 mg/ml in DMSO) was introduced into respective wells. Other wells containing DMSO and reference antibacterial drug served as negative and positive controls, respectively. The plates were incubated immediately at 37°C for 20 h. The activity was determined by measuring diameter of inhibition zone (in mm) showing complete inhibition. Growth inhibition was calculated with reference to positive control.[19]

RESULTS AND DISCUSSION

Antibiotic resistance is increasing worldwide in both outpatients as well as hospitalized patients. It varies according to geographic locales and is directly proportional to the use and misuse of antibiotics. Besides host and environmental factors, changes or mutation in genome of a pathogen, which occurred as a result of exposure to chemicals and antimicrobial agents (e.g., antibiotic), may lead to gene damage and emergence of drug resistant pathogen variants that could cause new disease. Despite newer antibiotic, continued selective antibiotic pressure and bacterial adaptation have resulted in a problem that can no longer

be ignored. Resistance can now be demonstrated against all available classes of antibiotics.[20-21]In last two decades, however, the problem has escalated as prevalence of antibiotic-resistant bacteria has increased and multi-drug-resistant strains have emerged in many species that cause disease in humans. There were no treatments available for infections caused by many of the antibiotic-resistant bacteria, and resistance to commonly used antibiotics was steadily increasing. Multiple drug-resistant organisms used in the current study are becoming common causes of infections in the acute and long-term care units in hospitals. *B. subtilis* has long been recognized as one of the most important bacteria that cause disease in humans. It was the leading cause of skin and soft tissue infections such as abscesses (boils), furuncles, and cellulites.

The question of fungal resistance to antibiotic drugs was considerably more SMEAs and was currently under evaluation. The question of drug resistance was complicated by the limitations in available susceptibility testing methodology and ability to distinguish between microbiological and clinical drug resistance.[22] The latter typically occurs when an inhibitory antifungal agent reached the limits of its activity in a host with a decreasingly efficient immune system. With the advent of SMEAs previously fatal infections can now be treated. However, as modern medicine continued to extend life through aggressive therapy of other life-threatening diseases such as cancer, there was an increasing population at risk for opportunistic fungal infections. Such patients represent a special challenge because they often were left with little host immune function. Therefore, chemotherapeutic agents should be fungicidal and not just fungistatic. The search continues for fungicidal agents that were nontoxic to the host. Research was also directed toward immunomodulating agents that can reverse the defects of native host immunity.[23]The corresponding mononuclear metal(II) SMEAswere studied as antimicrobial agents having bioaccessibility and bioavailability properties in different systems were interrelated, to abiotic (organic carbon) and biotic (uptake and metabolism).

Microbial Organometallic Assimilation: Bioaccessibility, Bioavailability and Bioaccumulation

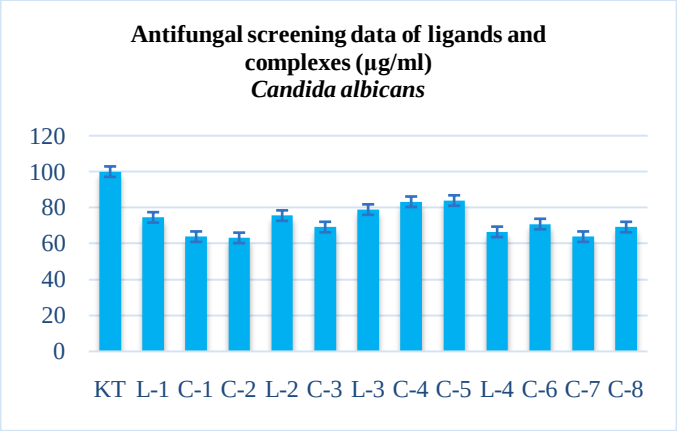
Enormous efforts have been devoted to the construction of molecular architectures by fusing the organic framework to metal centers through self-assembly processes. There has been continuous interest in the construction of stimuli-responsive SMEAs with diverse sizes, shapes, and symmetries to rationalize the criteria for molecular recognition and impart them on unique areas of applications, such as stereo selective guest encapsulation and molecular transporting devices. Although such a variety of metal-organic SMEAs architectures has been reported, those involving the use of noncovalent interactions other than those of hydrogen bonding, donor–acceptor, electrostatic, and hydrophobic-hydrophobic interactions that depend on the nature of the guests, which would be attractive for antimicrobial agents. Examples of such systems that can exhibited reversible host-guest association were also limited. The possibility of introducing responsive functionalities into the molecular rectangles, which might serve as models for the study of on-demand controlled guest capture and release systems, has also been explored. Additionally, the use of SMEAs as guest molecules, which have been shown to display antimicrobial capabilities, might lead to the design of a smart multi addressable molecular rectangle system that could capture and release specific guest molecules under different conditions to achieve proof-of-principle on-demand controlled drug delivery. Herein, the antimicrobial properties of SMEAs, molecular rectangles with different geometries, topologies and electronic properties were reported. Moreover, the encapsulation of various guest molecules was also investigated in detail to provide a proof-of-principle model for design of artificial drug delivery systems with modulation of drug release by ecological condition.

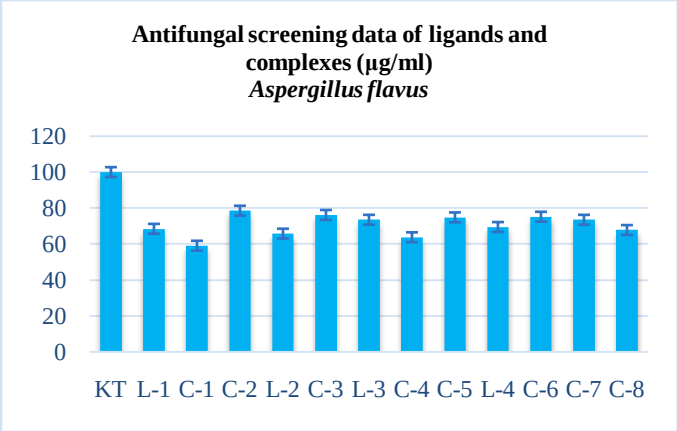
Microbes interact with metals and minerals in natural and synthetic environments, altering their physical and chemical state, with metals and minerals also able to affect microbial growth, activity and survival. In addition, many minerals are biogenic in origin, and the formation of such biominerals is of global geological and industrial significance, as well as providing important structural components for many organisms, including important microbial groups such as diatoms, foraminifera and radiolarian.[24] The free ligands and SMEAs were tested against various bacterial strains with the agar well diffusion method and Hanging drop method. The antifungal activity against various fungi was also tested and the results are presented as comparative manor graphically.

Antifungal Activity

The present communication describes antifungal activity of SMEA-1 to 8. The assessment of fungal toxicity of synthesized compounds is based on % age inhibition. Likewise in the present study, SMEAs showed different % of antifungal activities and provided evidences that they might indeed be potential sources of antimicrobial agents. Ligands (L-1 to L-4) and SMEA-1 to 8 were screened against different fungal strain i.e. Candida albicans, Aspergillus flavis and Candida glaberata. Ketoconazole was used as standard drug. The screening results presented graphically, in Fig. 5, and all ligands and SMEAs showing significant antifungal activities.

Some of them exhibited quite good activity, SMEAs C-4, C-7 and C-8 were found to be less effective against each species of tested fungi. Beside this C-4 and C-5 exhibited very good inhibition against Candida albicans. Biological screening data of C-2, C-3 and C-6, C-7 against aspergillus flavis depict high to moderate activity against fungi beside other ligands and SMEAs used with better physical properties. As well as, antifungal activity of C-1 against Candida glaberata reported very poorly efficient. Further, it has been concluded that C-5 and C-6 are more active against all fungal strain against them tests were performed than other ligands and SMEAs, indicated that they may act better antifungal activity in future. It has also been observed that C-1 and C-6 are more active than other SMEAs against Candida glaberata.





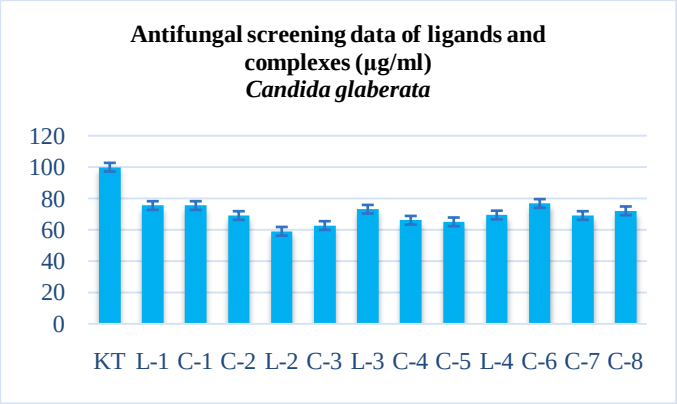


Figure 5. Antifungal activity of ligands (L-1 to L-4) and supramolecular entities of antibiotics (SMEA-C-1 to C-8)

It may be noted that SMEAs may be better antifungal agents. Finally, more and more experiments are needed to be conducted in order to understand the biological (including antifungal) activity of SMEA-1 to 8 and how can be modified better than ketoconazole without side effect.

It can be noted that SMEA-1 to 8 with more donor atoms showed the greater inhibitory effect on one or more types of fungus. The hydrogen of phenolic group is so reactive that it enabled the toxicants to combine with constituents of the living tissues, thus the toxicity of ligands was due alcoholic group and presence of phenyl groups in SMEA-1 to 8 bonded with metal atom was responsible for the rise of toxicity.

The antifungal activity of compounds has even more potency with respect to inhibition of microbes. Candida albicans, Aspergillus flavis and Candida glaberata as a fungal resistance to most of antibiotics showed a good sensitivity to both the series of derivatives.25 Resistance in Aspergillus and Candida increasingly investigated and reported because standards for susceptibility testing and associated breakpoints became available as a consequence of the increased use of antifungal compounds. Resistant infection can be encountered in the antifungal drug-exposed patient due to selection of intrinsically resistant species or isolates with acquired resistance belonging to species that were normally susceptible.

In both cases (ligands and SMEAs), resistance may be expected as any antimicrobial therapy was associated with a selection pressure and therefore risk of resistance. Resistance can, however, also be encountered in the antifungal drug-naïve patient and, again, can be due either to infection with intrinsically resistant species or to isolates with acquired resistance. Whereas resistance due to intrinsically resistant species can be diagnosed through correct species identification, detection of isolates with acquired resistance was more demanding and requires appropriate and carefully performed susceptibility testing and endpoint interpretation.

Antibacterial Activity

In general, earlier reported SMEA-1 to 8 derivatives were found more active against gram-positive bacteria than gram negative bacteria. This fact can be attributed to ease of permeation of SMEAs in formers owing to simplicity of cell membrane structure. The most probable reason for high antibacterial activity of metal derivatives than parent ligand was due to reduction in polarity of metal ion atom upon SMEAs action with ligand. This reduction in polarity was because of partial sharing of positive charge of metal ion with donor groups.

As a consequence lipophilic nature of central metal ion increased, which in turn enhanced permeation of SMEAs through lipid layer of cell membrane. Synthesized SMEAs showed significant antibacterial activity against *Escherichia coli, Bacillus subtilis*, and *Pseudomonas aeruginosa*. Imipenem was used as standard drug.[26] The screening results presented graphically in Fig. 6. The standard drug Imipenem was showed more significant antibacterial activity as compared to those having higher activity among reported SMEA i.e. C-5 and C-8 against Bacillus subtilis and C-1 and C-7 against Pseudomonas aeruginosa. As indicated in above graphs, C-2 showed very low antibacterial activity against Bacillus subtilis comparatively to other SMEAs. The C-2 and C-5 showed fairly good activity against Escherichia coli. C-1 and C-7 against Bacillus subtilis, and C-7 and C-8 against Pseudomonas aeruginosa, but not as comparable as reference drugs.

These results indicated that at same concentrations, C-1 of SMEAs has wider activity range than other SMEAs against Bacillus subtilis. Escherichia coli, Bacillus subtilis, and

Pseudomonas aeruginosa as bacterium resistance to most of antibiotics showed very good sensitivity to SMEAs.[26]

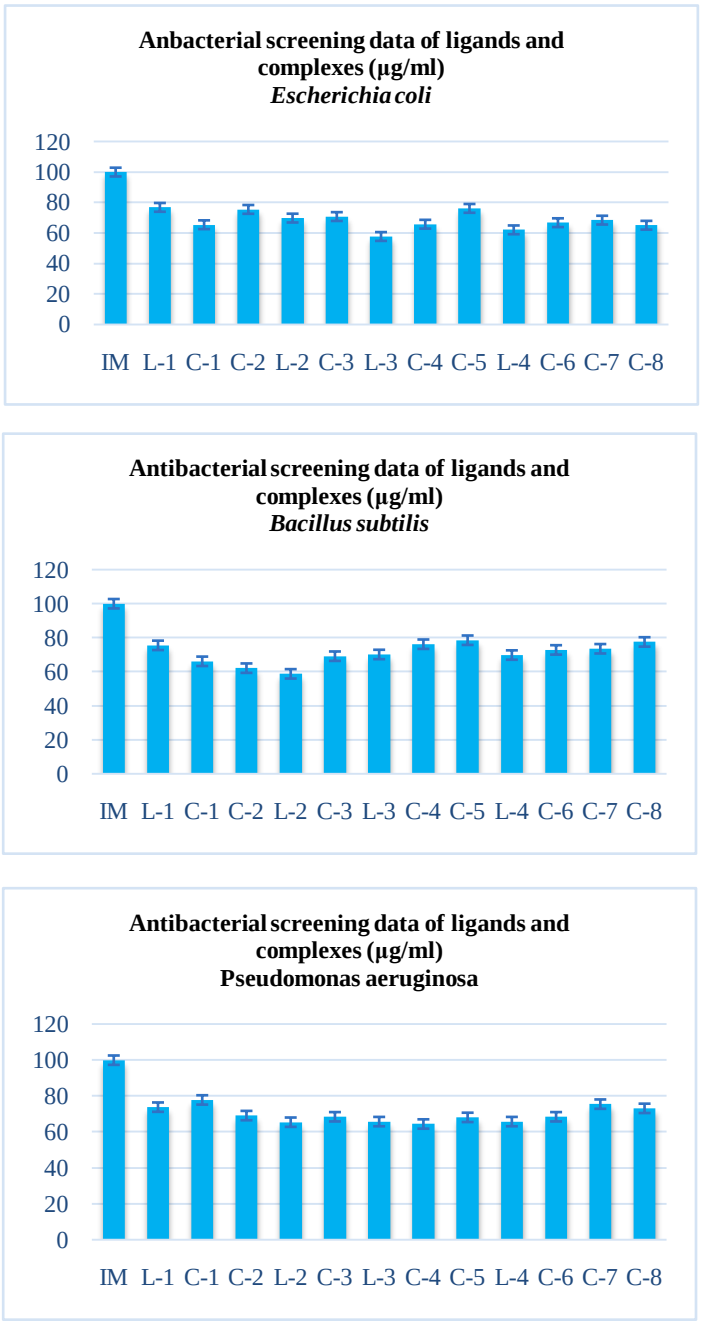


Figure 6. Antibacterial activity of ligands (L-1 to L-4) and supramolecular entities of antibiotics (SMEA-C-1 to C-8).

Achieved antibacterial test results were approximately in agreement. It can be noted that SMEAs with more phenyl groups showed the greater inhibitory effect on one or more types of bacteria as compared to alkyl groups SMEAs in the same position. The results compared with the standard drug (Imipenem) indicate that ligands and SMEAs are active however, their activity is less than that of the standard drug. The order of increasing antibacterial activities was C-4 < C-1, which matched with previously reported data for biological activity of supramolecular entities of antibiotics (SMEA-1 to 8). Bacteria are very resilient and have already developed resistance to many commonly used antibiotics.[27] Hydraphiles are synthetic amphiphiles that form ion-conducting pores in liposomal membranes. These pores exhibit open-close behavior when studied by planar bilayer conductance techniques. SMEAs co-administered with various antibiotics to strain of Escherichia coli, they enhanced the drug's potency. We report here potency enhancements at low concentrations of hydraphiles for structurally and mechanistically used antibiotics against Gram negative E. coli and Pseudomonas aeruginosa, as well as Gram positive Bacillus subtilis. Potency increases in correlation to ion transport function. The data presented here comport with the function of hydraphiles to enhance membrane permeability in addition to, or instead of, their known function as ion conductors.

CONCLUSION

The bioaccessibility, bioavailability, and bioaccumulation properties of inorganic metals in different systems are interrelated and abiotic (e.g., organic carbon) and biotic (e.g., uptake and metabolism). Modifying factors determined the amount of an inorganic metal that interacted at biological surfaces and that bond to and was absorbed across these membranes. A major challenge was to consistently and accurately measure quantitative differences in bioavailability between multiple forms of inorganic metals in the environment. The aim of this article was to summarize each category of earlier reported SMEAs in order to illustrate their physiological role, biochemical mechanism, environmental significance and bioremediation potential as antimicrobial biotransformation. During the following steps of metal utilization, the acquired metal was transferred through intracellular trafficking pathways which might include diverse storage compartments in order to be directed to cofactor assembly systems and to final microorganism targeting. Several of these used metals channeling routeswere described recently and provided first insights into later steps of metal assimilation that characterized an essential part of cellular metal homeostasis network. Solubility, concentration and fineness of the particle size of metal ion as well as the presence of bulkier organic moieties affected growth of organisms.[28] Microbes interacted with metals in natural and synthetic environments, altering their physical and chemical state able to affect their growth, activity and survival. Although antimicrobial studies were important, availability of drugs and their transport and release to site of action were equally important. To accomplish the controlled drug delivery functionalities, the first main strategy was to rigidifymolecular architecture of host from SMEAs to a rectangle, so that guest molecules would be better accommodated within cavity, which may lead to a more selective encapsulation of guests within a definite size and steric environment. The possibility of introducing responsive functionalities into molecular rectangles, which might serve as models for study of on-demand controlled guest capture and release systemswere explored (Fig. 7).

Solubility and concentration of compounds played vital role in ascertaining extent of inhibition. The inhibitory power of SMEA increased on increasing their concentration. The biological potency of supramolecular entities of antibiotics (SMEA) might be attributed to their ability to inactive various cellular enzymes of the microorganisms. Antimicrobials could attack various targets in microorganism, as a consequence of which organisms were either destroyed or had their growth inhibited. Since SMEA inhibited the growth of microorganisms, it was assumed that production of enzymes was being affected, and hencemicroorganisms were unable to utilize food for the intake of ion decreased and consequently their growth ceased.[29,30]

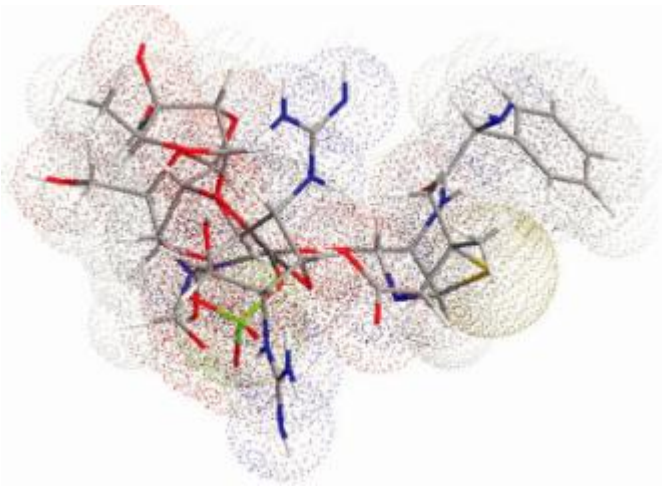


Figure 7. Antimicrobial potential: screening area of concerned atoms of supramolecular entities of antibiotics(SMEA)

These results exhibitedmarkedly an enhancement in activity on coordinationwith the metal ions against one or more tested bacterial strains*(Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa)*, and fungal strains*(Candida albicans, Aspergillus flavis and Candida glaberata)*. This enhancement in the activity was rationalized onthe basis of (L-1 to L-4) by possessing different linkages which imported in elucidatingthe mechanism of transamination reactions in ecology. It wasoutlined that the ligands with donor atoms might inhibit enzyme production, since theenzymes which required these groups for their activity appearto be especially more susceptible to deactivation by metalions upon the formation of coordinate bonds. This process of chelationthus increases the lipophilic nature of the central metal atom,which in turn favors its permeation through the lipid layerof the membrane. This in turn is responsible for increasingthe hydrophobic character and liposolubility of the moleculein crossing the cell membrane of the microorganism, and henceenhances the biological utilization ratio and activity of the testing drug/SMEAs.Antifungal resistance in *Candida and Aspergillus* may be either intrinsic or acquired and might be encountered in exposed antifungal drug but also is antifungal drug-naïve patient. Prior antifungal treatment conferred a selection pressure and notoriously raised awareness of possible resistance in patients failing therapy, thus calling for susceptibility testing. On the contrary, antifungal resistance in the drug-naïve patient was less expected and therefore more challenging. This was particularly true when it concerned pathogens with acquired resistance which cannot be predicted from species identification itself.[31]

Most of this attention had been devoted to study of antibiotic resistance in bacteria for several reasons: (i) bacterial infections are responsible for the bulk of community-acquired and nosocomial infections; (ii) the large and expanding number of antibacterial classes offers a more diverse range of resistance mechanisms to study; and (iii) the ability to move bacterial resistance determinants into standard well-characterized bacterial strains facilitates the detailed study of molecular mechanisms of resistance in bacterial species. One of the targets for novel antifungals under active investigation was fungal cell wall. Antifungal agents acting on this target were inherently selective. The subcellular mechanisms of their synthesis and assembly were used as potential targets to search for new antifungals and antimicrobialagentsand were identified as inhibitors acting at these levels. Development of new antibacterial agents, our research team were able to inhibit antibiotic resistant disease-causing microorganisms such as bacterial strain and fungal strain aggravates the emerging antibiotic resistance.

Specific methodology used as; *the aim of broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. MIC values are used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents. Agar dilution involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate. For broth dilution, often determined in 96-well microtiter plate format, bacteria are inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent. Growth is assessed after incubation for a defined period of time (16-20 h) and the MIC value is read. This protocol applies only to aerobic bacteria and can be completed in 3 d.*

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