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Antibacterial, Antifungal, Phytotoxic, and Genotoxic Properties of Two Complexes of Ag^{I} with Sulfachloropyridazine (SCP): X-ray Diffraction of $[Ag(SCP)]_n$

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We report the synthesis, characterization, antibacterial and antifungal activities, phytotoxicity, and genotoxicity of two new complexes of silver(I) with sulfachloropyridazine (SCP), one of which is heteroleptic with SCP and SCN^- ligands (Ag–SCP–SCN), the other of which is homoleptic (Ag–SCP); furthermore, the crystal structure of the homoleptic complex is disclosed. The heterocyclic N atom nearest to the Cl atom and the $N_{sulfonamide}$ atom could be coordination sites for the silver ion in the Ag–SCP–SCN complex. The Ag–SCP complex is a polymeric compound with metal–metal bonds, and the heterocyclic and sulfonamide N atoms are points of coordination for Ag^{I}. Both complexes showed activity against all the tested bacteria, and in the cases of *Escherichia coli* and *Pseudomonas aeruginosa*, the action was better than that of SCP. In all cases, both silver–SCP complexes showed better antifungal activity than SCP, which was inactive against the tested fungi. Notably, the activity against *P. aeruginosa*, a nosocomial multidrug-resistant pathogen, was better than that of the reference antibiotic cefotaxim. Both silver–sulfa complexes displayed moderate activity against the tested yeast, especially for *C. neoformans*, which is an important fact considering the incidence of cryptococcosis, mainly in immune-deficient patients. No chromosomal aberrations were observed with the *Allium cepa* test, which is auspicious for further study of these complexes as potential drugs.

Introduction

The treatment of infectious diseases still remains an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multidrug-resistant microbial pathogens. In spite of a large number of antibiotics and chemotherapeutics available for medical use, at the same time the emergence of old and new antibiotic resistance created in the last decades revealed a substantial medical need for new classes of antimicrobial agents.[1]

Historically, medicinal inorganic chemistry is rich in metal- and metalloid-based drugs, including Paul Erlich's organoarsenic compound for the treatment of syphilis, antiarthritic gold preparations, and diagnostic agents for magnetic resonance imaging (Gd, Mn, Fe) among others.[2] Silver has the most outstanding properties among all metals with antimicrobial activity because of its higher toxicity to microorganisms and lower toxicity to mammalian cells.[3] Silver compounds have been used as antibacterial agents since the middle ages, when silver nitrate was primarily used in the treatment of burns and wounds. In the 1940s, the introduction of penicillin drastically decreased the use of silver compounds for the treatment of bacterial infections, but recently, owing to the occurrence of infectious diseases caused by different pathogenic bacteria and mainly as a result of the growth of antibiotic-resistant bacteria, new metal–organic drugs with antibacterial activities have been synthesized and described.[4] Silver is effective against a broad range of Gram-negative and Gram-positive bacteria, fungi, and yeast. The pure metal is inactive; however, in the presence of moisture, silver readily ionizes to give silver cations, which show antimicrobial activity. Bacterial resistance to silver has been rarely reported.[5] Today, silver and silver nanoparticles (AgNPs) are used in healthcare, in the food industry, and in domiciliary applications, and they are commonly found in hard surfaces, materials, and textiles. The mechanisms of antimicrobial action of ionic silver have been studied, but there is little understanding of the interactions of AgNPs with microorganisms.[6] Interaction with the cell wall offers silver-containing species the possibility to penetrate the cell and to interact with compounds in its interior such as DNA or enzymes. Silver definitely has a great potential in the medical context as long as its concentration can be well controlled. We have to combat bacteria that are becoming more and more re-

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sistant to antibiotics. Silver and its compounds may well be a possible solution.[7]

Ag generally has no adverse effects for humans, and argyria (irreversible discoloration of the skin resulting from sub-epithelial silver deposits) is rare.[8] Many biologically active compounds used as medicinal drugs possess modified pharmacological and toxicological potentials if administered in the form of metal-based compounds. Various metal ions potentially and commonly used include cobalt, copper, nickel, and zinc, because of their capacity in forming low-molecular-weight complexes, and therefore, they are proven to be more beneficial against several diseases.[9] Recently, silver(I) complexes that showed good antimicrobial activity were synthesized by employing different ligands, such as saccharinate and tertiary monophosphanes,[10] hydroxymethyl derivatives of pyridine and benzimidazole,[11] and tryptophan[12] and pyridinedicarboxylate compounds[13] among others. Punctually about strains of *Pseudomonas aeruginosa* and *Escherichia coli*, a recently reported complex of silver with 2-mercaptothiazolidine and triphenylphosphine showed strong activity. The significant high activity of the complex against both microbial strains is in agreement with its ability to bind DNA.[14]

The synthesis of metal sulfanilamide compounds has received much attention owing to the fact that sulfanilamides were the first effective chemotherapeutic agents to be employed for the prevention and cure of bacterial infections in humans.[15] N-Substituted sulfonamides are still among the most widely used antibacterial agents in the world, mainly because of their low cost, low toxicity, and excellent activity against bacterial diseases.[16] They are the drugs of choice for the treatment of chancroid, nocardiosis, and acute urinary tract infections caused by microorganisms such as *E. coli*, *Proteus mirabilis*, and others. They can be used in combination with other drugs in the treatment of otitis, meningitis, toxoplasmosis, recurrent and chronic urinary tract infections, and diarrhea, among other diseases.[17] Sulfonamides exert their antibacterial action by the competitive inhibition of the enzyme dihydropterase synthetase because of its similarity with *p*-aminobenzoic acid, a factor required by bacteria for folic acid synthesis.[18]

The interest in metal–sulfanilamide derivatives was stimulated by the successful introduction of a Ag^{I} sulfadiazine complex to prevent bacterial infections during burn treatment of both humans and animals, and this complex is still in current use.[19] Sulfachloropyridazine (SCP), 4-amino-*N*-(6-chloro-3-pyridazinyl)-benzenesulfonamide, $C_{10}H_9ClN_4O_2S$ (Figure 1), is a sulfonamide used as an antimicrobial for urinary tract infections and in veterinary medicine, alone and in combination with trimethoprim.[20]

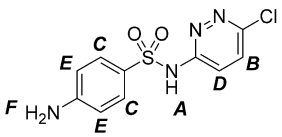

Figure 1. Sulfachloropyridazine (SCP): 4-amino-*N*-(6-chloro-3-pyridazinyl)benzenesulfonamide, $C_{10}H_9ClN_4O_2S$; labels indicate the notation used for SCP and their derivatives for ^{1}H NMR spectroscopic assignments.

As a continuation of our work on metal complexes of sulfa drugs, in this paper we report the synthesis, characterization, antibacterial and antifungal activities, phytotoxicity, and genotoxicity of two complexes of silver(I) with SCP (one heteroleptic with the SCP and SCN^- ligands and the other homoleptic with the SCP ligand); furthermore, the crystal structure of the homoleptic complex is examined.

Results and Discussion

Synthesis

The obtained complexes, white solids slightly soluble in water but sufficiently soluble in DMSO to allow microbiological tests to be performed, presented elemental analysis values in agreement with the calculated values.

Spectroscopic measurements

Selected IR spectral data of SCP and its silver complexes

SCP shows characteristic NH_2 bands corresponding to asymmetric and symmetric stretching at 3496 and 3395 cm^{-1}, respectively.[21] The third band that appears at 3316 cm^{-1} is assigned to N–H stretching of the sulfonamide moiety. This band is not observed in the other compounds, probably as a result of deprotonation. A shift in this group of bands to higher energy can be observed. This fact is consistent with that observed for other metal–sulfa drug complexes.[22] This parameter may indicate the coordination of the metal ion with the amino group, although some authors believe that it can also be due to hydrogen-bonding interactions of the group.[23] The bands attributed to the SO_2 vibrations are shifted to higher energies in both complexes, which is suggestive of an interaction of the $-SO_2-N-$ group with the metal ion.[24]

The bands attributed to the pyridazine nucleus decrease both in number and in intensity in the complexes with respect to the free ligand, which is suggestive of an interaction with the N-heterocyclic atoms. In the Ag–SCP–SCN complex, the appearance of two bands at 487 and 455 cm^{-1} correspond to the Ag–S and Ag–N interactions. Similarly, the appearance of bands at 2101 and 2087 cm^{-1} assigned to ν(CN) indicate the coordination of the thiocyanate with the silver ion through the S atom.[25] According to the observed changes in the vibrational IR spectral data, it is possible to suggest that the $N_{sulfonamide}$, N_{amide}, and $N_{heterocycle}$ atoms would be coordination points for the Ag^{I} ions with the sulfa moiety, and in the Ag–SCP–SCN complex, the thiocyanate ion is bonded to the silver ion through the S atom.

NMR spectroscopy

^{1}H NMR

The signal of the amide proton [H(A), $\delta = 11.69$ ppm in SCP] is absent in the ^{1}H NMR spectrum of the complex, which indicates that the sulfa moiety is deprotonated in the complex.[26]

The ^{1}H NMR spectrum of the Ag–SCP–SCN complex shows broadening of almost every signal (Figure 2).

Almost all of the ^{1}H NMR resonances suffer upfield shifts, similarly to other silver complexes,[27] but they are most noticeable for the following: H(B), 7.74 ppm in SCP and 7.59 ppm in the complex ($\Delta\delta = -0.15$ ppm); H(F), 6.11 ppm in SCP and 5.78 ppm in the complex ($\Delta\delta = -0.33$ ppm); and H(C), 6.62 ppm in SCP and 6.52 ppm in the complex ($\Delta\delta = -0.10$ ppm).

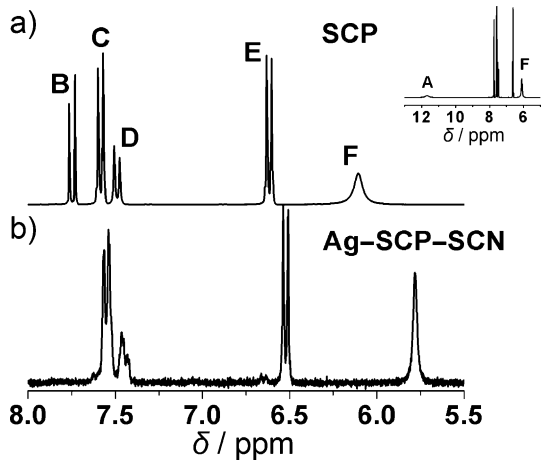

Figure 2. ^{1}H NMR spectra of a) SCP and b) Ag–SCP–SCN in [D_6]DMSO; inset: $\delta = 13$–5 ppm range.

^{13}C NMR

The most notable changes were observed in the pyridazine ring signals, particularly those assigned to the atoms of C bonded to the sulfonamide group ($\delta = 154$ ppm in the ligand, $\delta = 161$ ppm in the complex) and to H(B) ($\delta = 150$ and 146 ppm, respectively). These results, which are in agreement with those of the vibrational spectra, allow us to suggest that the heterocyclic N atom nearest to the Cl atom and the $N_{sulfonamide}$ atom could be coordination sites for the silver ion, without discarding the N atom of the amine group.

Crystal structure determination of the homoleptic complex, Ag–SCP

Figure 3 represents (ORTEP-3)[27] the asymmetric unit of the polymeric complex [Ag_2-SCP]$_n$; the molecular structure thereof is viewed through the symmetry operator (#) $-2-x, 1-y, 1-z$. The sulfachloropyridazine acts like a bidentate ligand,[28,29] in which the two nitrogen atoms are responsible for coordinating two metal centers. The nitrogen N2 atom is deprotonated, which provides a negative charge that is neutralized by coordination with Ag1. Moreover, the N3 atom of the pyridazine function is coordinated to the silver Ag2 atom neutrally. The Ag^I ions show a linear coordination geometry with angles of 180° for N3–Ag2–N3# and N2–Ag1–N2#2. Symmetry operators (#) $-2-x, 1-y, 1-z$; (#2) $-1-x, 1-y, 1-z$.

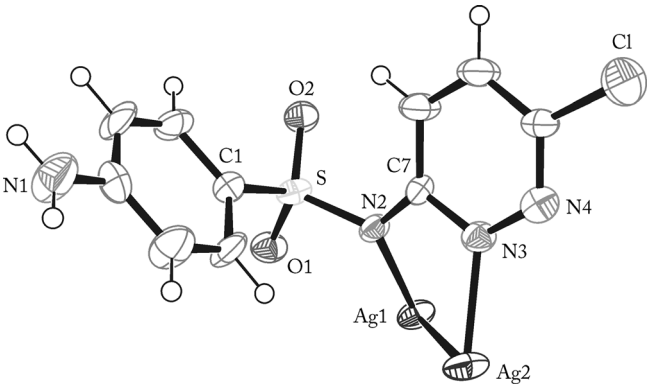

Figure 3. ORTEP representation of the until cell of [Ag_2-SCP]$_n$: thermal ellipsoids are drawn at the 50% probability level.

The lengths of the Ag–N bonds are 2.105(6) and 2.134(6) Å for N2–Ag1 and N3–Ag2, respectively, which are slightly shorter than those reported in the literature.[30–33] In contrast, the length of the Ag1–Ag2 bond is 2.8897(3) Å. The values found are within the expected range of bond lengths for Ag–N and Ag–Ag, according to published data.[34] Selected distances and angles are listed in Table 1.

Table 1. Selected bond lengths and angles for [Ag_2-SCP]$_n$.[a]

Bond length [Å]		Bond angle [°]	
Ag1–N2	2.105(6)	N2–Ag1–N2#2	180.000(1)
Ag1–N2#	2.105(6)	N2–Ag1–Ag2	74.34(15)
Ag1–Ag2	2.8897(3)	N3–Ag2–N3#	105.66(15)
S–N2	1.625(7)	N3#–Ag2–Ag1	180.0(4)
N3–N4	1.344(9)	Ag1–Ag2 Ag1#4	102.21(16)
N2–C7	1.349(9)	Ag2#3–Ag1–N2#2	77.79(16)
N3–C7	1.366(9)		

[a] Symmetry transformations used to generate equivalent atoms: (#) $-2-x, 1-y, 1-z$; (#2) $-1-x, 1-y, 1-z$; (#3) $1+x, y, z$; (#4) $-1+x, y, z$.

The angle between the chlorine atom of the pyridazine function and the nitrogen atom of the primary amine of the aromatic ring together with the sulfur atom (Cl–S–N1) form a distorted V configuration[35,36] with a value of 88.14(8)°. Substantially equal to that observed in the free ligand, the angle for the same atoms is 85.12(2)°. On the basis of this information, we believe that the spatial twist caused by this configuration can be crucial in obtaining the 1D polymeric structure (Figure 4).

Biological evaluation

Antimicrobial activities

The emergence of resistance in bacterial strains has become one of the prime concerns of the 21st century,[37] whereas the increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance have encouraged the search for new antifungal agents or effective combinations of

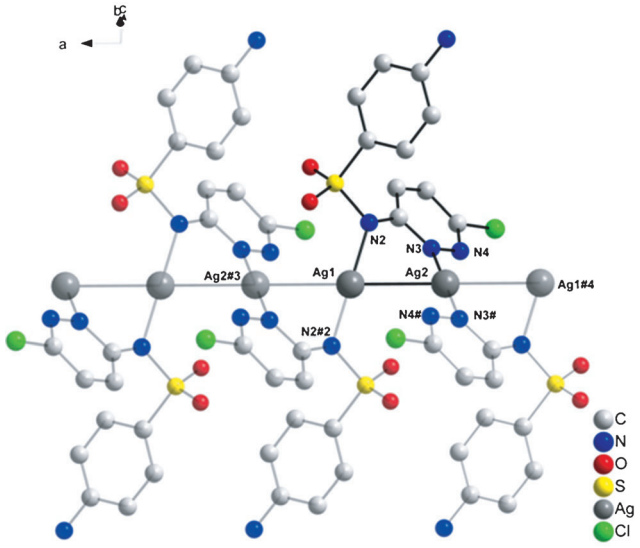

Figure 4. Polymeric structure of $[Ag_2\text{-SCP}]_n$: Hydrogen atoms are omitted for clarity. Symmetry transformations used to generate equivalent atoms: (#) $-2-x, 1-y, 1-z$; (#2) $-1-x, 1-y, 1-z$; (#3) $1+x, y, z$; (#4) $-1+x, y, z$.

existing drugs.[38] Results of the antibacterial and antifungal assays with SCP and its silver complexes Ag–SCP and Ag–SCP–SCN are listed in Tables 2 and 3, respectively. Examples of some Ag^I complexes against the same as or similar to our tested strains are included for comparative purposes.

Both complexes showed activity against all tested bacteria, and in the cases of *E. coli* and *P. aeruginosa*, the action was better than that of SCP alone and of the same order of magnitude or better than other silver complexes.[10,41,42] Notably, both complexes showed a minimal inhibitory concentration (MIC, $\mu g\,mL^{-1}$) against *P. aeruginosa*, which is a leading nosocomial pathogen that may become multidrug resistant;[43] this is much better than other tested compounds and even better than the reference antibiotic cefotaxime.[39] In addition, the silver content was also the lowest of the tested substances, so potential risks of argyria[44] are decreased.

In contrast to antibacterial agents, only a few antimetabolites are available for use against pathogenic fungi. In spite of the fact that fungal organisms have a pathway for folate, as well as bacteria, inhibitors of folate metabolism such as sulfonamides[45] are not effective in the treatment of *C. albicans* infections. Among the reasons for this fact, the mentioned differences between the nature of the key enzymes in the folic acid biosynthetic pathways of fungus and bacteria[46] and the impermeability of the membrane to the same drugs could be a factor.[38] With respect to metal complexes of sulfa drugs, in addition to its well-known antibacterial activity, silver sulfadiazine was recently reported to possess strong antifungal properties, which makes it a clinically widely used topical agent for the treatment of wound and burn infections.[47] In all cases, both silver–SCP complexes showed better antifungal activity than SCP alone, which was inactive against the tested fungi. Both silver–sulfa complexes displayed moderate activity against the tested yeast, especially for *C. neoformans*, which could be of interest considering the increase in antifungal drug resistance and the fact that incidence of cryptococcosis has increased considerably, mainly owing to diverse causes of immunodeficiency.[48]

The mechanism of action of silver cations is not yet completely understood. Silver cations bind to bacteria cell surfaces and interact with enzymes and proteins important for cell-wall synthesis. Silver can also affect cell respiration, transport, and metabolism, as well as DNA, RNA, and subcellular organelle structures.[42] Furthermore, it is probable that the inhibition of phosphomannose isomerase, a key enzyme in the biosynthesis of yeast cell walls, imparts good antifungal activity to the same sulfa–Ag complexes.[27b] A weak-bonding Ag ligand would play a key role in the antimicrobial activities of silver(I) complexes.[41] Despite the high activity found for silver nitrate against both microbial strains, the main problem with the use of this salt in medicine is its high solubility in water, with its simultaneous very low lipophilicity. Thus, the silver ions immediately released after silver nitrate application are chemically consumed and are rapidly inactivated through the formation of chemical complexes by chloride within a few hours.[14] Recent results showed that Ag–mordenite exerted effective antifungal action owing to the release of silver ions from the zeolite matrix, which acted directly on the walls of the microor-

Table 2. MIC values of sulfachloropyridazine (SCP), its Ag^I complexes Ag–SCP and Ag–SCP–SCN, and $AgNO_3$ acting against human pathogenic bacteria. Published MIC values of silver complexes **1**–**3** are given for comparison.

Complex	MIC [$\mu g\,mL^{-1}$] (Ag [μM])[a]						
	E. coli [Gram−]		*P. aeruginosa* [Gram−]		*S. aureus* [Gram+]		
	ATCC25922	Mach1 (Invitrogen)	ATCC27853	PAO1 (Invitrogen)	ATCC29213	ATCC25923	ATCC33591
SCP	>23.5	–	>8.3	–	3.20	–	–
Ag–SCP	10.00 (25.5)[b]	–	5.20 (13.2)[b]	–	5.20 (13.2)[b]	–	–
Ag–SCP–SCN	20.00 (84.6)	–	2.10 (8.90)[b]	–	5.20 (22.0)	–	–
$AgNO_3$	6.77 (39.9)	–	8.79 (51.7)	–	2.20 (13.0)	–	–
1[c]	(57.9–229.2)	–	–	–		(14.5–229.2)	(29.0–114.6)
2[d]	–	(40–400)	–	(150)		–	–
3[e]	(79.6)	–	(79.6)	–		(79.6)	–

[a] Minimal inhibitory concentration; reference values of cefotaxime for *E. coli* ATCC25922: MIC = 0.03–0.12 $\mu g\,mL^{-1}$; *P. aeruginosa* ATCC27853: MIC = 8–32 $\mu g\,mL^{-1}$; *S. aureus* 29213: MIC = 1–4 $\mu g\,mL^{-1}$[39]. [b] A remarkable result. [c] Complex **1**: silver(I) saccharinate complexes with tertiary monophosphanes.[10] [d] Complex **2**: silver(I) complexes with 2-mercaptothiazole and its derivatives and triphenylphosphine.[14] [e] Complex **3**: silver(I) complex with ibuprofen.[40]

Table 3. MIC/MFC values of sulfachloropyridazine (SCP), AgSCN, the SCP silver(I) complexes Ag–SCP and Ag–SCP–SCN, and $AgNO_3$ acting against human opportunistic pathogenic fungi. Published MIC values of silver complexes **1**–**4** are given for comparison.

Sample[a]	MIC [$\mu g\,mL^{-1}$] (Ag [μM])[b]											
	SCP	AgSCN	Ag–SCP	Ag–SCP–SCN	$AgNO_3$	Amp	Ket	Terb	**4**[c]	**5**[d]	**6**[e]	**7**[f]
C. albicans	>250	31.25/	31.25 (79.6)/	62.50 (263.6)/	6.36 (37.4)/	0.78	6.25	1.56	(70.1)	(21–52)	(13.9–226.4)	(3.2–5.1)
ATCC10231		62.50	125.0	125.0	12.72							*C. albicans* C316
C. tropicalis	>250	31.25/	31.25 (79.6)/	62.50 (263.6)/	12.72 (74.9)/	1.56	6.25	0.78	–	–	(13.9–209.0)	–
C131		62.50	125.0	125.0	25.44							
S. cerevisiae	>250	31.25/	31.25 (79.6)/	62.50 (28.44/263.6)/	6.36 (4.04/37.4)/	0.78	3.12	3.12	(70.1)	–	–	–
ATCC9763		62.50	125.0	125.0	12.72							
C. neoformans	>250	15.60/	15.60 (39.9)[g]/	15.6 (65.9)[g]/	3.18 (18.7)/	0.78	1.56	0.39	–	–	–	–
ATCC32264		62.50	31.25	125.0	12.72							
A. fumigatus	>250	31.25/	31.25 (79.6)/	31.25 (131.8)/	25.44 (149.7)/	3.12	12.5	0.78	–	–	–	–
ATCC26934		62.50	62.50	125.0	25.44							
A. flavus	>250	31.25/	31.25 (79.6)/	31.25 (131.8)/	12.72 (74.9)/	0.78	6.25	0.78	–	–	–	(5.3–19.4)
ATCC9170		125.0	125.0	125.0	12.72							*A. flavus* C1150
A. niger	>250	31.25/	62.50 (159.4)/	31.25 (131.8)/	25.44 (149.7)/	0.78	6.25	1.56	(140.2)	–	–	(6.2–22.9)
ATCC9029		125.0	125.0	125.0	25.44							*A. niger* C418
M. gypseum	>250	62.50/	62.50 (159.4)/	125 (527.3)/	12.72 (74.9)/	6.25	12.5	0.006	–	–	–	–
C115		62.50	125.0	125.0	12.72							
T. rubrum	>250	62.50/	62.50 (159.4)/	62.5 (263.6)/	6.36 (37.4)/	6.25	12.5	0.003	–	–	–	–
C113		62.50	62.50	125.0	6.36							
T. mentagrophytes	>250	62.50/	62.50 (159.4)/	62.5 (263.6)/	12.72 (74.9)/	6.25	12.5	0.006	–	–	–	–
ATCC9972		62.50	62.50	125.0	12.72							

[a] ATCC = American Type Culture Collection (Rockville, MD, USA); C = CEREMIC, Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina. [b] MIC: minimal inhibitory concentration; MFC: minimal fungicidal concentration; Amp: amphotericin B; Ket: ketoconazole; Terb: terbinafine. [c] Complex **4**: dinuclear silver(I) *N*-acetylglycinate complexes.[41] [d] Complex **5**: silver(I) complexes of hydroxymethyl derivatives of pyridine and benzimidazole.[11] [e] Complex **6**: Ag–N-heterocyclic carbene complexes.[42] [f] Complex **7**: silver complexes of sulfa drug derivatives.[27b] [g] A remarkable result.

ganisms, and this was more effective than the free silver ions in solution.[49] The Ag–SCP and Ag–SCP–SCN complexes were more efficient than $AgNO_3$ as antibacterial agents (considering the % of Ag) but the opposite took place as antifungal agents. However, the silver complexes have the advantage of a slow release of silver cation, which prevents inactivation[14] and cytotoxicity of the free Ag^{I} ions.[50]

*Plant genotoxicity test (*Allium cepa *test)*

The development of new drugs imposes the need to assess their potential toxicity in experimental models. The *Allium cepa* test was selected to evaluate the potential risks of the Ag–SCP and Ag–SCP–SCN complexes. Since 1938, *Allium cepa* L (common onion) biological material has been widely used in laboratory tests, because of the rapid growth of its roots and the response of its genetic material to the presence of potentially cytotoxic and genotoxic substances in liquid media.[51] The *Allium cepa* species has been frequently used to determine the cytotoxic, mutagenic, and genotoxic effects of several substances, and it is considered the standard organism for quick tests, as it shows a high correlation with mammal test systems.[52] Another advantage of this test system is the presence of an oxidase enzyme system that is essential for promutagen evaluations.[53] The results of phytotoxicity (as a change in root length, expressed in cm) and mitotic index (MI, ‰) of the Ag–sulfa complexes evaluated with the *Allium cepa* test are listed in Tables 4 and 5, respectively.

The mitotic index was determined by scoring more than 5000 cells (1000 cells per slide).[54] The mitotic index was calculated as the number of dividing cells (taking into account the following mitotic phases: meta-, ana-, and telophase) per 1000 observed cells.[55] SCP, $AgNO_3$, and KSCN, tested for comparative purposes, did not show genotoxic properties with the *Allium cepa* test (data not shown). The correlation between the data of each set of doses of the sulfa drug and effects in the *Allium* test was evaluated by linear regression (Figures 5 and 7). Statistical significance was considered at 5% ($p < 0.05$).[56]

Table 4. Phytotoxicity of sulfachloropyridazine (SCP) and its silver(I) complexes evaluated using the *Allium cepa* test.

Conc. [$g\,(L \times 10^4)^{-1}$]	Δ [cm][a]		
	SCP	Ag–SCP	Ag–SCP–SCN
0	2.97 ± 0.30	0.92 ± 0.12	1.20 ± 0.59
12.5	2.92 ± 0.34	0.86 ± 0.09	1.18 ± 0.22
62.5	3.03 ± 0.58	0.60 ± 0.12	1.00 ± 0.23
250.0	2.31 ± 0.89	0.41 ± 0.16	0.93 ± 0.21
625.0	2.96 ± 0.54	0.31 ± 0.19	0.65 ± 0.19
1250.0	2.46 ± 0.61	0.19 ± 0.11	0.27 ± 0.10

[a] Phytotoxicity is expressed as the change in root length (Δ); data represent the mean ± SD of $n = 5$ independent experiments; $K_2Cr_2O_7$ ($\delta =$ 1 ppm) was used as positive control: $\Delta = 1.01 \pm 0.31$ cm.

Ag–SCP–SCN showed 50% inhibition of root length from the negative control at the concentration of 73.6 $mg\,L^{-1}$, whereas Ag–SCP showed this value at 62.1 $mg\,L^{-1}$. Both values were calculated with the linear regression equations shown in Figure 5. Root degeneration was not observed over the entire assayed range. According to these results, Ag–SCP–SCN did

Table 5. Mitotic index of sulfachloropyridazine (SCP) and its silver(I) complexes evaluated by using the *Allium cepa* test.

Conc. [g (L×10^4)$^{-1}$]	MI [%][a]		
	SCP	Ag–SCP	Ag–SCP–SCN
0	36.56±6.29	55.60±4.22	36.56±6.29
12.5	36.60±4.22	39.60±5.50	37.00±6.63
62.5	37.40±5.59	38.00±1.73	47.00±3.16
250.0	35.33±3.72	28.40±3.71	35.90±7.09
625.0	32.20±4.27	26.00±3.08	27.83±6.08
1250.0	34.83±6.11	23.60±8.56	12.00±1.79

[a] Data represent the mean±SD of $n=5$ independent experiments; $K_2Cr_2O_7$ ($\delta=1$ ppm) was used as positive control: $\Delta=40.50\pm6.09$.

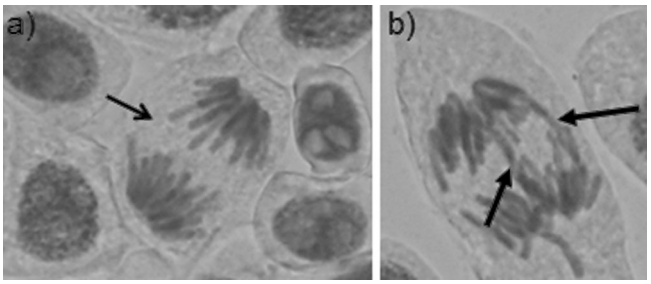

Figure 6. Chromosomal aberration observed in *Allium cepa* meristematic cells exposed to chemical agents: a) normal anaphase (this work) and b) anaphase with chromosome bridge.[57]

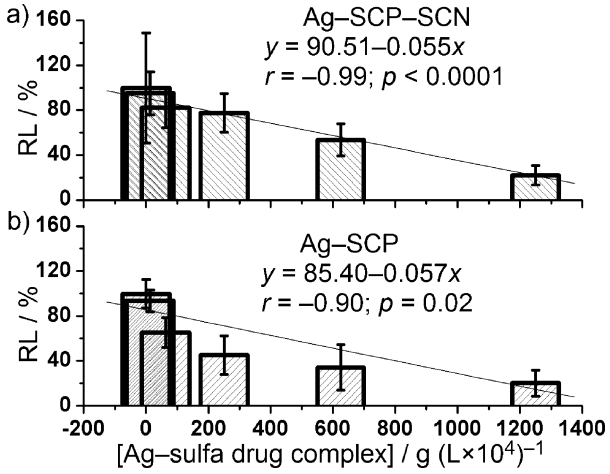

Figure 5. Effect of the concentration of a) Ag–SCP–SCN and b) Ag–SCP on the root length of bulbs of *Allium cepa* L. Root length (RL), as percentage of negative control, is plotted as a function of sulfa drug concentration. Linear regression ($y=a+bx$) is shown, weight given by data±SD error bars.

not show phytotoxic effects on onions in the concentration range that demonstrated antibacterial and antifungal activities. Ag–SCP also did not show phytotoxic effects in the range of antibacterial MIC. Most of the antifungal MICs (60%) were non-phytotoxic concentrations, and the rest were in the limit. Chromosomal aberrations (CA) were not observed with the complexes or with their reagents ($AgNO_3$, KSCN, SCP). Figure 6 shows an example of a normal anaphase and another with a chromosome bridge (i.e., CA).

The inhibition of mitotic activities is often used to trace cytotoxic substances. The mitotic index (MI), characterized by the total number of dividing cells in a cell cycle, was used as a parameter to assess the cytotoxicity of several agents. The cytotoxicity levels of an agent can be determined by the increase or decrease in the MI. MIs significantly lower than that expressed by the negative control can indicate alterations deriving from the chemical action in the growth and development of exposed organisms. In contrast, MIs higher than that expressed by the negative control are the result of an increase in cell division, which can be harmful to the cells, as it can lead to disordered cell proliferation and even to the formation of tumor tissues.[53] The cytotoxic limit value, calculated from the MI of the treated sample/MI of the control)×100,[58] points out that a decrease in the MI below 22% of that of the control causes lethal effects on test organisms, whereas a decrease below 50% (cytotoxic limit value) usually has sublethal effects.[59] However, the inhibition of division without the observation of chromosome aberrations is a very promising result for anticancer therapy, as it first leads to blocking the development of cancer.[60] Drugs that interfere with the normal progression of mitosis belong to the most successful chemotherapeutic compounds currently used for anticancer treatment.[61] Targeting the progression of mitosis has been proven to be an efficient strategy for anticancer therapy.[62] Silver complexes with antibacterial and antitumor activity at the same time have been reported recently,[10] and although it is not the aim of this work, it is important to note this property, which may be useful in further studies on these complexes.

Figure 7 shows the cytotoxic coefficient of the Ag–sulfa complexes, calculated as MI of the treated sample/MI of the negative control. A value of 100 mg L^{-1} was found for the cyto-

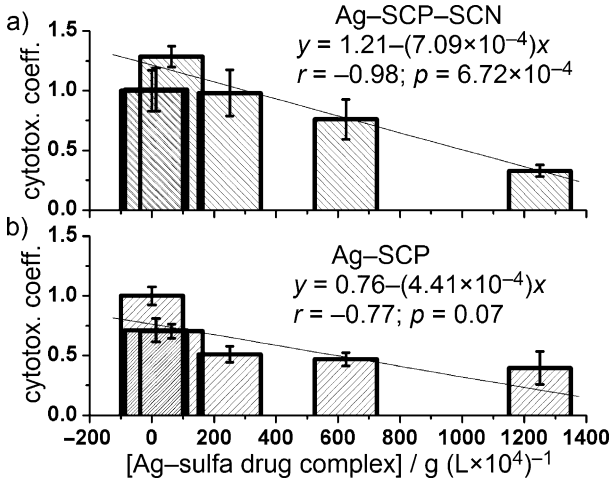

Figure 7. Effect of the concentration of a) Ag–SCP–SCN and b) Ag–SCP on the mitotic index (MI) of meristematic cells of *Allium cepa* L. Cytotoxic coefficients, calculated as $(MI_{sample})/(MI_{control})$, are plotted as a function of the concentration of each Ag–sulfa complex. Linear regression ($y=a+bx$) is shown, weight given by data±SD error bars.

toxic coefficient = 0.5 for the Ag–SCP–SCN complex, that is, a decrease in MI by 50%, whereas the respective value for Ag–SCP was near 60 mg L^{-1}. Both values were calculated with the linear regression equations shown in Figure 7. Similarly to that observed with the phytotoxic effects, the MICs of the two complexes as antibacterial agents fell outside risk values. Some MICs values of the Ag–SCP complex as an antifungal were close to 50% inhibition of the MI. Summarizing, the Ag–SCP and Ag–SCP–SCN complexes showed decreased root elongation of *Allium cepa* L, as did $AgNO_3$. No chromosomal aberrations were observed with the *Allium cepa* test, which is auspicious for further study of these complexes as potential drugs. Among the damages caused by chemical agents to exposed organisms, the genotoxic and mutagenic effects are worrying because of their capacity to induce genetic damage, which can lead to several health problems and also affect future generations, as these alterations can be inheritable; therefore, it is very important that a compound that could be a medicine does not cause CA.[53]

Conclusions

Herein we report the synthesis, characterization, antibacterial and antifungal activities, phytotoxicity, and genotoxicity of two complexes of silver(I) with SCP (one heteroleptic with SCP and SCN^- ligands and the other homoleptic with the SCP ligand); furthermore, the crystal structure of the homoleptic complex was investigated. The heterocyclic N and sulfonamide N atoms could be coordination sites for the silver ion, without discarding the N atom of the amine group. Ag–SCP is a polymeric compound with metal–metal bonds. Both complexes showed activity against all bacteria tested, and in the cases of *E. coli* and *P. aeruginosa*, the action was better than that of SCP alone. In all cases, both silver–SCP complexes showed better antifungal activity than SCP, which was inactive against the tested fungi. Notably, the action of both Ag–sulfachloropyridazine complexes against *P. aeruginosa*, a nosocomial pathogen multidrug resistant, was better than that of the reference antibiotic cefotaxime. Both silver–sulfa complexes displayed moderate activity against the tested yeast, especially for *C. neoformans*, an important fact considering the incidence of cryptococcosis, mainly in patients with immunodeficiency. No chromosomal aberrations were observed with the *Allium cepa* test, which is auspicious for further study of these complexes as potential drugs.

Experimental Section

Chemistry

General: All reagents and solvents were purchased from Sigma. IR spectra, in the range between 4000 and 200 cm^{-1}, were recorded with a Bomem M 102 FTIR spectrophotometer by using the KBr pellet technique for both complexes. ^1H NMR and ^{13}C NMR spectra of the Ag–SCP–SCN complex were recorded with a Bruker Avance 300 NMR spectrometer at ambient probe temperature (≈25 °C) with nominal operating frequencies of 300.1 and 50.3 MHz, respectively. All chemical shifts (δ) are quoted in parts per million (ppm). The chemical shift scales are internally referred to the non-deuterated DMSO singlets at $\delta = 2.71$ and 39.39 ppm for ^1H and ^{13}C NMR, respectively. Normal 2D techniques were used, including HSQC and HMBC, for the assignment of the ^{13}C NMR signals.

Synthesis: The homoleptic complex Ag–SCP was synthesized according to a previously reported procedure.[63] The heteroleptic complex Ag–SCP–SCN was synthesized by mixing of SCP (0.284 g, 1 mmol; Sigma), which was previously dissolved in water at pH 9–10 by adding 1 M NaOH, with a suspension obtained by mixing equimolar aqueous solutions of $AgNO_3$ and KSCN. The mixture was stirred in the absence of light for 20 min, centrifuged, washed several times with water and then with ethanol, and dried in the dark. Elemental analysis was performed with a Carlo Erba EA1108 elemental analyzer. Ag assessments were performed by volumetric determination with ammonium thiocyanate (USP29). Compound Ag–SCP is a white solid (0.29 g, 74%). Suitable crystals for crystallographic study were obtained in a gradient of 9.7 mL DMSO and 0.3 mL H_2O. FTIR (KCl disk): $\tilde{\nu} = 3459, 3355, 3063, 1572, 1294, 1119, 962, 556\ cm^{-1}$. Elemental analysis calcd (%) for $C_{10}H_8ClN_4O_2SAg$ (AgSCP): C 30.672, H 2.059, N 14.308, Ag 27.5; found: C 30.858, H 2.089, N 14.048, Ag 27.8. Compound Ag–SCP–SCN is a white solid (0.24 g, 79%). FTIR (KCl disk): $\tilde{\nu} = 3563, 3458, 3356, 3070, 2102, 2087, 1577, 1297, 1127, 963, 563\ cm^{-1}$. Elemental analysis calcd (%) for $C_{13}H_{10}ClN_7O_3S_4Ag_4$ ($Ag_3[Ag(SCN)_3(SCP)]\cdot H_2O$): C 17.20, H 1.11, N 10.80, Ag 45.5; found: C 17.59, H 1.15, N 11.01, Ag 46.6.

Crystallography

A Bruker CCD X8 Kappa APEX II diffractometer operated with a graphite monochromator and MoK_α radiation ($\lambda = 0.71073$ Å) was used for X-ray structure analyses. The molecular crystal structure of the ligand and the Mo complex was solved by direct methods with SHELXS.[64] The final structure was refined with SHELXL[64] with anisotropic displacement parameters for all non-hydrogen atoms; hydrogen atoms were refined isotropically as riding atoms at their theoretical ideal positions, with the exception of the hydrogen atoms that were located for the purpose of a discussion of interactions and bonding. Drawings were made with DIAMOND for Windows.[65] More detailed information on the structure determination is given in Table 6.

Biology

Antibacterial assays: Agar dilution tests were used to determine the minimal inhibitory concentration (MIC) of the antimicrobial agent against *E. coli* ATCC25922, *Staphylococcus aureus* ATCC29213, and *P. aeruginosa* ATCC27853. Most of the steps in the procedure for performing the agar dilution susceptibility tests were based on recommendations from the National Committee for Clinical Laboratory Standards.[66] A suspension of the ligands and silver complexes in sterile water and DMSO (≤5%) was incorporated into 10 mL of Mueller-Hinton agar and poured into Petri dishes. The pH of each batch of medium was in the range from 7.2 to 7.4. The dilution scheme for each antimicrobial agent covered a range of 200.0–0.25 (μg sulfonamide) mL^{-1}. The same process was performed with a water dilution of $AgNO_3$. The standard strains used were *E. coli* ATCC25922 and *S. aureus* ATCC29213. In each case, a suspension of ~1×10^8 colony-forming units per mL (CFU mL^{-1}) in Trypticase soy broth was prepared and diluted (1:20) in physiological serum. The suspension (2 μL) was inoculated as a spot of 5–8 mm in diameter on the agar surface containing the indicated dilutions of the antimicrobial agent and on the control dish containing no antimicrobi-

Table 6. Crystal data and structure refinement for $[Ag(SCP)]_n$.

Parameter	$C_{10}H_8AgClN_4O_2S$
M_r [Da]	391.58
T [K]	293(2)
radiation, λ [Å]	0.71073
crystal system	monoclinic
space group	$P2_1/n$
unit cell dimensions	
a [Å]	5.7794(6)
b [Å]	18.722(3)
c [Å]	11.5966(14)
α [°]	90
β [°]	94.292(7)
γ [°]	90
V [Å^3]	1251.3(3)
Z	4
calculated density [g cm^{-3}]	2.079
absorption coefficient [mm^{-1}]	1.993
F(000)	768
crystal size [mm]	0.112×0.057×0.035
θ range [°]	2.07–26.87
index ranges	$-7 \le h \le 6$
	$-23 \le k \le 21$
	$-14 \le l \le 14$
collected reflections	10614
unique reflections	2661 [R(int) = 0.1180]
completeness to θ_{max} [%]	98.8
absorption correction	Gaussian
max. and min. transmission	0.9975 and 0.9006
refinement method	full-matrix least-squares on F^2
data/restraints/parameters	2661/0/175
goodness-of-fit on F^2	0.920
final R indices [$I \ge 2\sigma(I)$]	$R1$ = 0.0496
	$wR2$ = 0.0753
R indices (all data)	$R1$ = 0.1715
	$wR2$ = 0.1047
largest diff. peak and hole [e Å^{-3}]	0.558 and −0.533

al agent. All tests and inoculation on each dish were run in duplicate.

Antifungal assays: The microorganisms used for fungistatic evaluation were purchased from ATCC or were clinical isolates from CEREMIC (identified with the capital letter C), Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina. Yeasts: *Candida albicans* ATCC10231, *C. tropicalis* C131, *Saccharomyces cerevisiae* ATCC9763, *Cryptococcus neoformans* ATCC32264; hialohyphomcetes: *Aspergillus flavus* ATCC9170, *A. fumigatus* ATCC26934, *A. niger* ATCC9029; dermatophytes: *Trichophyton mentagrophytes* ATCC9972, *T. rubrum* C113, *Microsporum gypseum* C115 were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C. For yeasts, cell suspensions in sterile distilled water were adjusted to give a final concentration of 1×10^3 viable yeast cells per mL.[67] For filamentous fungi, the strains were maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to reported procedures[67] and adjusted to 1×10^3 spores with colony-forming ability per mL. The minimal inhibitory concentration (MIC) of each compound was determined by using broth microdilution techniques according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards NCCLS)[68] for yeasts (M27-A2) and for filamentous fungi (M38A). MIC values were determined in RPMI-1640 (Sigma, St Louis, Mo, USA) buffered to pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid (MOPS). Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth for the rest of the fungi. For the assay, stock solutions of pure compounds were twofold diluted with RPMI from 250 to 0.98 μg mL^{-1} (final volume = 100 μL) and a final DMSO concentration ≤1%. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control, for which sterile water was added to the well instead. Endpoints (MIC) were defined as the lowest concentration of drug resulting in total inhibition of visual growth relative to the growth in the control wells containing no antifungal. The minimal fungicidal concentration (MFC) was determined by plating a duplicate 5 μL from each clear well of MIC determinations onto a 150 mm SDA plate. After 48 h at 37 °C, MFCs were determined as the lowest concentration of each compound showing no growth in the plates. Amphotericin B (Janssen Pharmaceutica, Belgium), Ketoconazole (Sigma Chem. Co. St Louis, MO, USA), and Terbinafine (Novartis, Bs. As., Argentina) were used as positive controls. Both MIC and MFC were confirmed by two replicates.

*Plant genotoxicity test (*Allium cepa *test)*: For this test, which was performed by following standard procedures,[55] equal-sized young bulbs of common *Allium cepa* were used. Stock solutions of 0.125 g L^{-1} of each complex were prepared by dissolving the compounds (0.0625 g) in DMSO (7 mL) and commercial mineral water in sufficient quantity to 250 mL. Stock solutions of SCP and KSCN were similarly prepared. Aliquots from these solutions were taken to perform the experiments, which covered a 0.125–0.00125 g L^{-1} sulfonamide range. Onion bulbs (seven per dose) were kept in mineral water for 48 h, then exposed to the silver complexes and ligand solutions (SCP and KSCN) for 24 h, and next, the onions were placed in mineral water for 24 h (recovery time). The noninterference of $AgNO_3$ was checked by a dose–response curve. We were interested in the influence of the silver cations on the onions in the culture medium of the onions. The silver(I) ion concentration released from the complexes was not sufficient for precipitation of AgCl into the mineral water in which the onions were grown.

The roots were then fixed in 1:3 acetic acid/ethanol solution for 24 h and finally stored in 70% ethanol. The roots growing in mineral water were used as a negative control, whereas treatment with $K_2Cr_2O_7$ (1–5 mg L^{-1} in mineral water) represented a positive control. The length of the roots as an index of toxicity and modifications in root consistency and shape (formation of tumors, hook roots, twisted roots) were observed as macroscopic parameters. The microscopic parameter was the mitotic index (five slides, 1000 cells per slide) to evaluate cellular division rate.

Chromosome preparation and staining: Root tips were hydrolyzed in 1 M HCl at 60 °C for 10 min before staining in Schiff's reagent (from Sigma: pararosaniline 1%, sodium metabisulfite 4%, in HCl 0.25 M) for 15 min. After the root caps were removed from well-stained root tips, 1 mm of the meristematic or mitotic zones was immersed in a drop of 2% orceine in 45% acetic acid (which was used to stain the chromosomes) on a clean slide and squashed into single cells. A Globe light microscope was used with ×640 magnification. Photographs of selected preparations were taken with an OLYMPUS BX40 optical microscope coupled to a digital camera (OLYMPUS D-560 ZOOM).

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Keywords: allium test · antibacterial agents · antifungal agents · silver · sulfonamides

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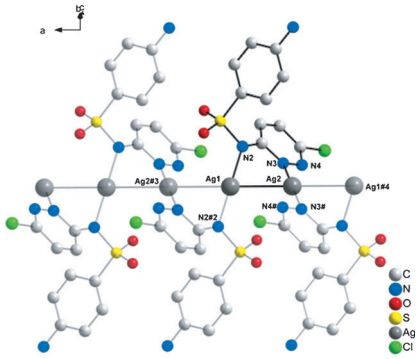

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Antibacterial, Antifungal, Phytotoxic, and Genotoxic Properties of Two Complexes of Ag^{I} with Sulfachloropyridazine (SCP): X-ray Diffraction of $[Ag(SCP)]_n$