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Nickel(II)-PPh_3 complexes with ONS and ONN chelating thiosemicarbazones: synthesis and inhibition potential on influenza A viruses

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Abstract: Tetra-coordinated nickel(II) complexes of two ONS (**1**, **2**) and seven ONN (**3a**–**3g**) chelating 2-hydroxy-3-methoxy-benzaldehyde thiosemicarbazones were synthesized. The dibasic ligands and complexes bearing PPh_3 as a coligand were characterized by means of analytical and spectroscopic data. Cytotoxic activities of the ligands and nickel(II) complexes were determined using the MTT assay in vitro against MDCK cells, and then all the compounds were tested on influenza virus replication by plaque assays. The compounds showed GI_{50} values varying from concentrations of 15.9 up to 161.8 μ g/mL for MDCK cells. The plaque assays indicated that one ONS (**1**) and two ONN (**3c** and **3d**) chelate structures have considerable antiviral effects on influenza A viruses at lower concentrations than the GI_{50} values for MDCK cells. The ligands and other complexes did not show any inhibitory effects on influenza virus plaque formation. The effects of the compounds on the influenza virus and structure–antiviral activity relationships were discussed based on the donor atoms and S-alkyl substituents.

Key words: Thiosemicarbazone, Ni(II) complexes, cytotoxic activity, antiviral, influenza A viruses

1. Introduction

Influenza A viruses belong to the family *Orthomyxoviridae*. These viruses cause frequent epidemics and sometimes pandemics affecting the human population all over the world. Over the past century, three major influenza A virus pandemics have taken place: the Spanish flu (H1N1) in 1918, the Asian flu (H2N2) in 1957, and the Hong Kong flu (H3N2) in 1968.[1] Influenza A viruses have a wide range of host specificity and the capability to infect many birds and mammal species in nature.[2] These viruses cannot be easily transmitted to new host species. Occasionally avian influenza viruses can directly infect humans, such as in the recent outbreak of highly pathogenic H5N1 avian influenza in Southeast Asia.[3,4] A current concern is that an avian influenza strain will directly acquire the ability for sustained infection of humans or reassortment with circulating human strains and cause a new pandemic. Recurrent infections of influenza viruses in the human population are largely due to the continual changes occurring in the antigenic properties of virus surface glycoproteins.[5,6] In particular, the changes of the viral hemagglutinin proteins enable the virus to avoid the immunological defense of the host organism.[7] Consequently, the control of influenza by vaccination is not completely effective. Today, M2 blockers and NA inhibitors are being used against influenza A virus infections.[8,9] Studies have shown that the vast majority of influenza virus types in circulation are resistant to M2 blockers, adamantane derivatives.[10]

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Therefore, NA protein inhibitors have greater importance in the treatment of influenza virus infections. NA and HA, the other membrane protein, are major surface glycoproteins and determine the subtype of influenza viruses.[11] Influenza A viruses are reported to have 18 different variants of HA molecules (H1–18) and 11 different variants of NA molecules (NA1–11) (https://www.cdc.gov/flu/about/viruses/transmission.htm).[12] HA protein, which is important for host specificity, binds to sialic acid residues of cell receptors and provides adsorption and penetration of the virus into the cell.[13,14] NA, on the other hand, is an enzyme that removes sialic acid residues of cell receptors.[15,16] NA facilitates the passage through the mucus layer covering the respiratory tract mucosa and cuts the sialic acid bonds, allowing the virus to be released from the cells.[17] It has been shown to fail to replicate defective artificial viruses in terms of NA protein.[18] Because of its enzymatic activity, NA protein is an important target for the inhibition of viral replication. NA inhibitors currently in use are the analogues of sialic acid residue.[19] These molecules show their inhibitory effects on different influenza strains by blocking the release of the newly synthesized viruses from host cells by inhibiting the NA enzyme.[20] However, influenza virus HA and NA surface antigen proteins are the most variable proteins resulting from mutations. Therefore, viruses can frequently develop resistance to these drugs.[20–23] Amino acid substitutions, particularly in the sialic acid binding pocket of NA protein, cause resistance to NA inhibitors. The viruses carrying amino acid substitution at positions 136 (Q136K) and 275 (H275Y) of NA protein show resistance to zamamivir and oseltamivir, respectively.[24,25] Therefore, the development of new antiviral drugs may be effective against the influenza A virus and this process requires continuous work.

Compounds having nitrogen and sulfur atoms such as thiosemicarbazones are an important class of compounds owing to their wide range of biological and pharmaceutical activities.[26] Their biological activity has been thought to improve selectively in certain biological systems, due to their ability to chelate formations with metal ions.[27] For this reason, they could be promising compounds as chelating inhibitors of influenza virus endonuclease.[28] Thiosemicarbazones and their transition metal complexes have biological activities such as antitumor,[29] antibacterial, antifungal,[30,31] and antiviral[32] effects.

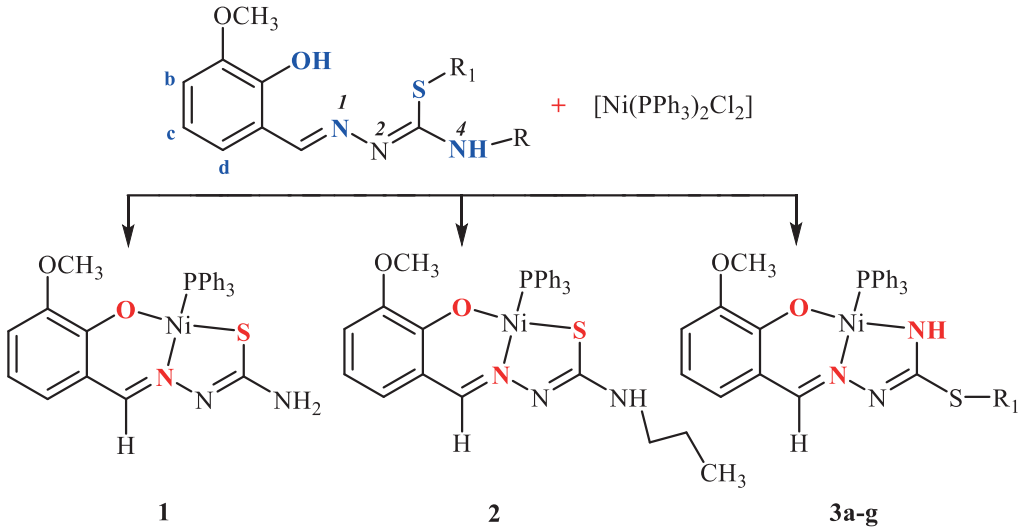
The therapeutic potential of thiosemicarbazones was first reported in the mid-1940s with a study describing the effect of some thiosemicarbazones against *Mycobacterium tuberculosis* in in vitro assays.[33] In 1950, benzaldehyde thiosemicarbazone was presented as an antiviral agent that was effective against the *Vaccinia* virus in fertile eggs and mice.[34] Among the compounds studied, methisazone (N-methyl-isatin-β-thiosemicarbazone) was clinically used as a synthetic antiviral agent for the treatment of smallpox.[35,36] Thematic studies related to the S-alkyl derivative of thiosemicarbazones are very rare.[37]

In this paper, the synthesis, characterization, cytotoxic, and antiviral properties of nine nickel(II)-PPh_3 complexes with 2-hydroxy-3-methoxy-benzaldehyde-thiosemicarbazone ligands having an ONS ($\mathbf{L^{1,2}}$) or ONN ($\mathbf{L^{3a-3g}}$) donor set are presented (Scheme). To investigate the probable biological potentials of the dibasic ligands, ONS and ONN chelate structures, and the effect of alkyl chain length, the ligands and complexes were tested on influenza virus replication by plaque assays and their cytotoxic effects were determined on MDCK cells.

2. Results and discussion

2.1. Chemistry and spectroscopic data

The free ligands, obtained as crystalline powders, were dissolved in common organic solvents. The reactions of the thiosemicarbazone ligands with $[Ni(PPh_3)_2Cl_2]$ in equivalent amounts resulted in the tetracoordinated



Scheme. The synthesis of the complexes (R/R_1: H/H (**L**^{1}); C_3H_7/H (**L**^{2}); H/CH_3 (**L**^{3a}); H/C_2H_5 (**L**^{3b}); H/C_3H_7 (**L**^{3c}); H/C_4H_9 (**L**^{3d}); H/C_5H_{11} (**L**^{3e}); H/C_6H_{13} (**L**^{3f}); H/C_7H_{15} (**L**^{3g})).

nickel(II) complexes (Scheme). The compositions of **1**, **2**, and **3e**–**3g** were compatible with the expected formula, [Ni(L)(PPh_3)]. Recrystallizations of **3a**–**3d** resulted in the composition of [Ni(L)(PPh_3)]·HCl in like ONN complexes of analogous thiosemicarbazones.[38,39] This formation could be explained by finding the place of a HCl molecule in the crystal lattice due to the smaller atomic radius of nitrogen (N^4) than the sulfur atom. The complexes, **1**, **2**, and **3a**–**3g**, were in the form of fine crystals, soluble in alcohols and chlorinated hydrocarbons. The dark red-colored complexes were stable in air. The crystals lost their brightness after 1 month, but the chemical compositions of the complexes did not change for months. These chemical stability results were obtained by means of TLC and electronic spectra.

The stretching bands of the OH and N^2H (for **L**^{1}) groups of the ligands were observed in the regions of 3338 cm^{-1} and 3307 cm^{-1}, respectively. The combination bands arising from stretching vibration of N^2=C and bending vibrations of NH_2 were in the 1623–1538 cm^{-1} region.[40] After the complex formation, the ν(OH) and δ(NH) (one of the δ(NH) bands in **L**^{1} and also **L**^{2}) were no longer observed. In a similar manner, the ν(OH) and one of the ν(N^4H) bands belonging to the complexes (**3a**–**3g**) were absent due to the deprotonation of **L**$^{3a-3g}$. The observed frequency differences of the ν(NH), δ(NH), and ν(CH=N) resulting from coordination reaction indicated a complex formation and these changes were not very clearly descriptive for the suggested structures. However, the presence of ν(PPh_3) bands at 1438, 1100, and 700 cm^{-1} was evidence of the structures intended for complexes **1**, **2** and **3a**–**3g**.[41]

^{1}H-NMR spectra provided additional information for the ligand and complex structures. While the imine protons (CH=N) of the ONS ligands (**L**^{1} and **L**^{2}) had one singlet peak, ONN ligands (**L**$^{3a-3g}$) showed two singlets at nearly 8.40 and 8.25 ppm, indicating syn-anti isomerism.[42] NMR spectra showed clear evidence related to the complex formation. After coordination of **L**$^{1-3}$, the signals of the removed two protons were not observed in the complex spectra (Figures 1 and 2). Specifically, ligands **L**^{1} and **L**^{2} were displayed in the form of a thione tautomer. Therefore, the protons separated by complexation are on N^2 nitrogen, and they were signaled at 9.86 (for **L**^{1}) and 9.75 (for **L**^{2}) ppm.

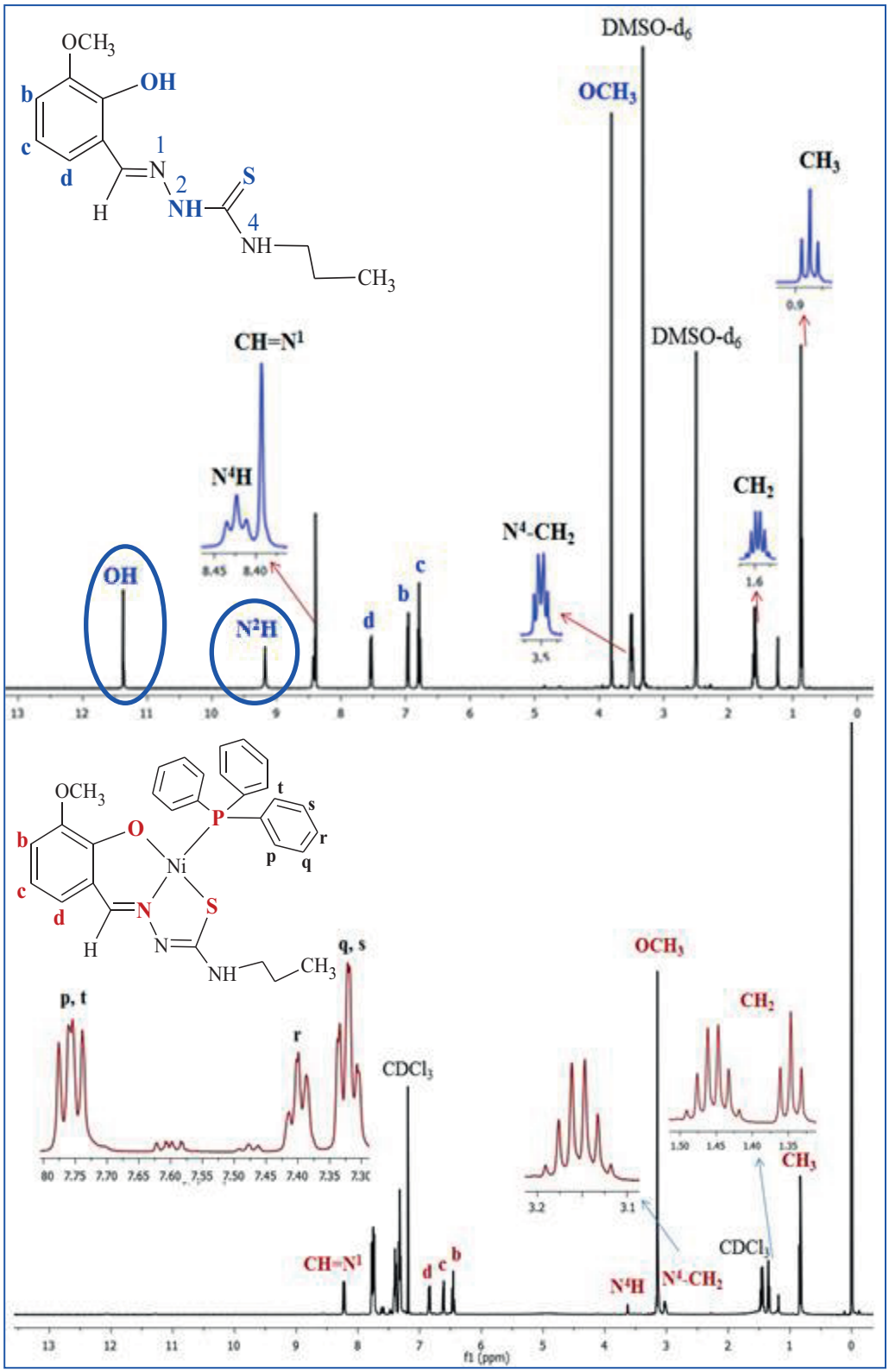


Figure 1. ^{1}H NMR spectra of the ONS ligand ($\mathbf{L^2}$) and complex (**2**).

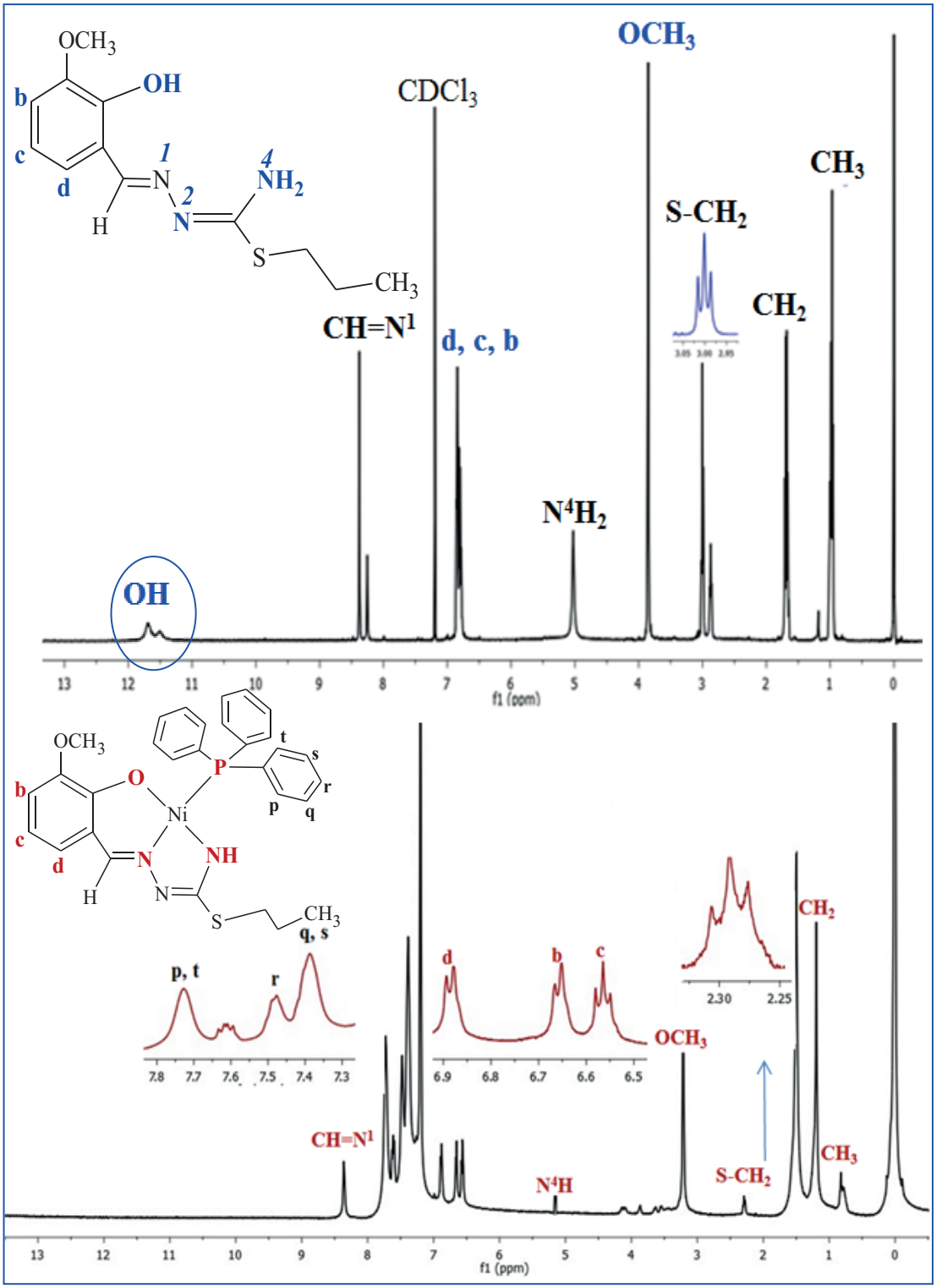


Figure 2. ^{1}H NMR spectra of the ONN ligand ($\mathbf{L}^{\mathbf{3c}}$) and complex (**3c**).

By coordination of the ligands to nickel(II), the imine protons of the ONS ligands were recorded at 8.17 ppm with shifting to lower frequencies. The anti-isomer peak of the ONN-chelating ligands disappeared and the syn-isomer peaks shifted more or less to higher frequencies. For N^4H protons, the changes in the chemical shift values associated with complex formation were not regular due to the differences in the molecular conformations depending on the elongation of the alkyl chain.

Other evidence of the formation of **1**, **2**, and **3a**–**3g** were 3-OCH_3 proton signals, which appeared at ca. 3.85 ppm in the NMR spectra of the ligands, considerably shifted to the 3.36–3.14 ppm region in the complexes.

In the ^{31}P NMR spectra of the complexes, the bands observed in the range of 29.56–30.37 ppm were attributed to the P atom in the basal plane.[43]

2.2. Cytotoxicity and antiviral studies

All of the compounds synthesized as mentioned above were initially tested for in vitro cytotoxic activity against MDCK cells. The MTT assay was used for determining cytotoxicity and % cell death was calculated at different concentrations of the compounds using the following formula:

$$\% \text{ Cell Death} = [(\text{Control OD} - \text{Treated OD}) / \text{Control OD}] \times 100\%$$

In Figure 3A, only the toxicity effects of complexes **1**, $\mathbf{3^c}$, and $\mathbf{3^d}$ on MDCK cells are given. The percentage of cell death was calculated with respect to the untreated control and the % cell death values were plotted to the concentrations of compounds (Figure 3A). The concentration of each compound for 50% cell growth inhibition (GI_{50} value) against MDCK cells was determined from the respective dose-response curve given in Figure 3B (Table).

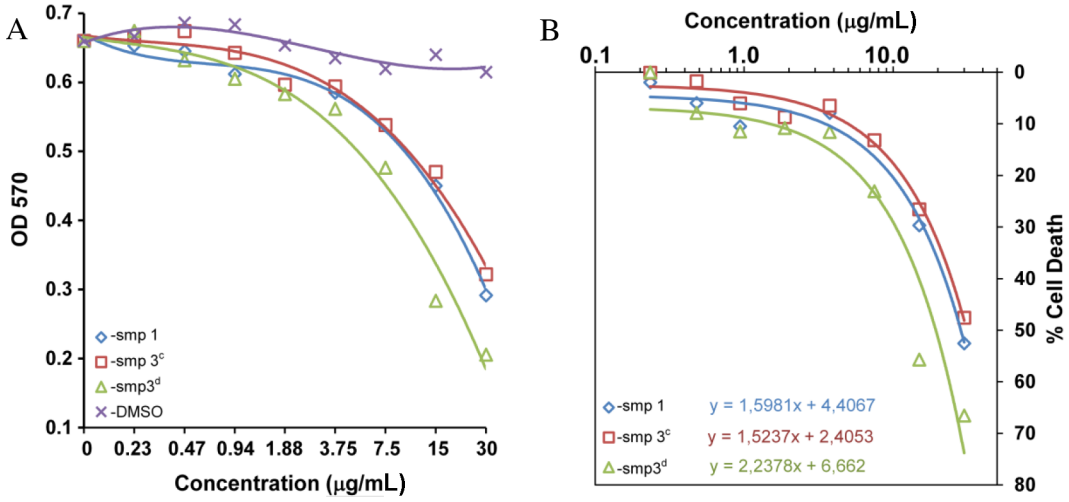


Figure 3. The cytotoxicity of **1**, $\mathbf{3^c}$, and $\mathbf{3^d}$ on MDCK cells. A) MTT reduction by complexes, B) % cell death curves derived from MTT.

In general, the complexes showed higher levels of toxicity effects on MDCK cells compared to ligands. While the lowest toxicity was observed with ligand $\mathbf{L^{3f}}$, complex $\mathbf{3^g}$ was found most toxic for the cells. This was respectively followed by complexes $\mathbf{3^a}$ and **2** with GI_{50} values of 17.4 μg/mL and 18.3 μg/mL.

The antiviral activities of nickel(II) complexes and ligands were revealed by using plaque inhibition tests with influenza A viruses. The compounds were tested at lower concentrations than those of the GI_{50} values.

Table. Cytotoxicity of compounds against MDCK cell lines in terms of GI_{50} values (μg/mL).

Ligand	GI_{50}	Complex	GI_{50}
$\mathbf{L^1}$	45.8	1	28.5
$\mathbf{L^2}$	72.0	2	18.3
$\mathbf{L^{3a}}$	66.2	3^a	17.4
$\mathbf{L^{3b}}$	48.9	3^b	17.4
$\mathbf{L^{3c}}$	36.7	3^c	31.2
$\mathbf{L^{3d}}$	27.8	3^d	19.4
$\mathbf{L^{3e}}$	65.4	3^e	20.8
$\mathbf{L^{3f}}$	>100	3^f	21.4
$\mathbf{L^{3g}}$	77.3	3^g	15.9

Three complexes, **1**, $\mathbf{3^c}$, and $\mathbf{3^d}$, among 18 compounds tested on influenza virus plaque formation showed inhibitory effects on influenza virus plaque formation (Figure 4). Plaque formation by influenza A viruses was almost completely inhibited by these complexes at 10 μg/mL concentration. These three complexes inhibited influenza virus plaque formation by more than 50% at 5 μg/mL concentration. In contrast, other complexes and ligands were found not to be effective against influenza A viruses at 10 μg/mL concentrations (Figure 5).

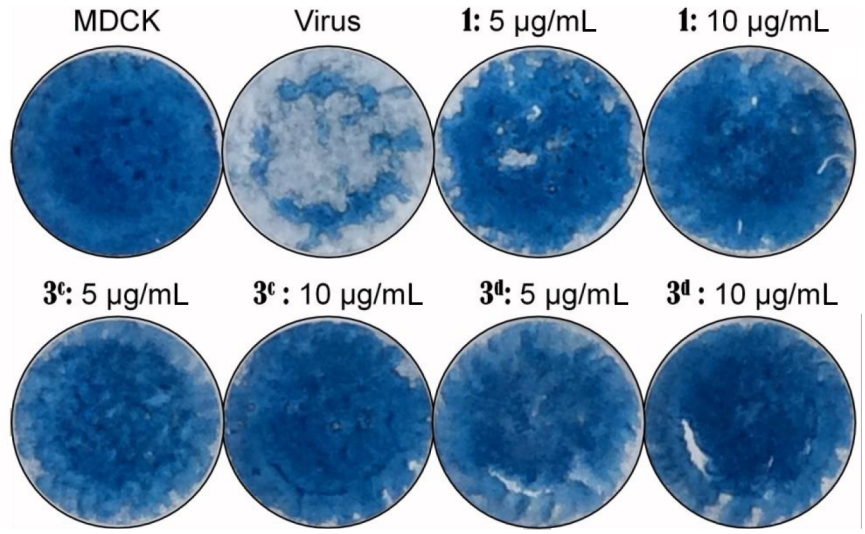


Figure 4. The anti-influenza virus activity of the complexes (**1**, $\mathbf{3^c}$, $\mathbf{3^d}$). Monolayers of MDCK cells were infected with influenza A virus (WSN). After 30 min of infection, virus inoculums were removed and monolayers were overlaid with 0.6% agarose-maintenance medium, with or without complexes. After 2–3 days of incubation, monolayers were fixed and stained with Amido black dye solution.

2.3. Conclusions

As is known, the 2-hydroxy-arylidene-thiosemicarbazones were ONS (N^4-substituted) or ONN (S-substituted) chelating ligands and methyl or phenyl groups were the generally preferred substituents.[38,42,44] In this study, new nickel(II)–triphenylphosphine complexes with S-methyl, ethyl, propyl, butyl, pentyl, hexyl, and heptyl alkylated 2-hydroxy-3-methoxy-benzaldehyde thiosemicarbazones were synthesized and characterized. These tetracoordinated complexes with long alkyl chains such as hexyl and heptyl were first reported and their

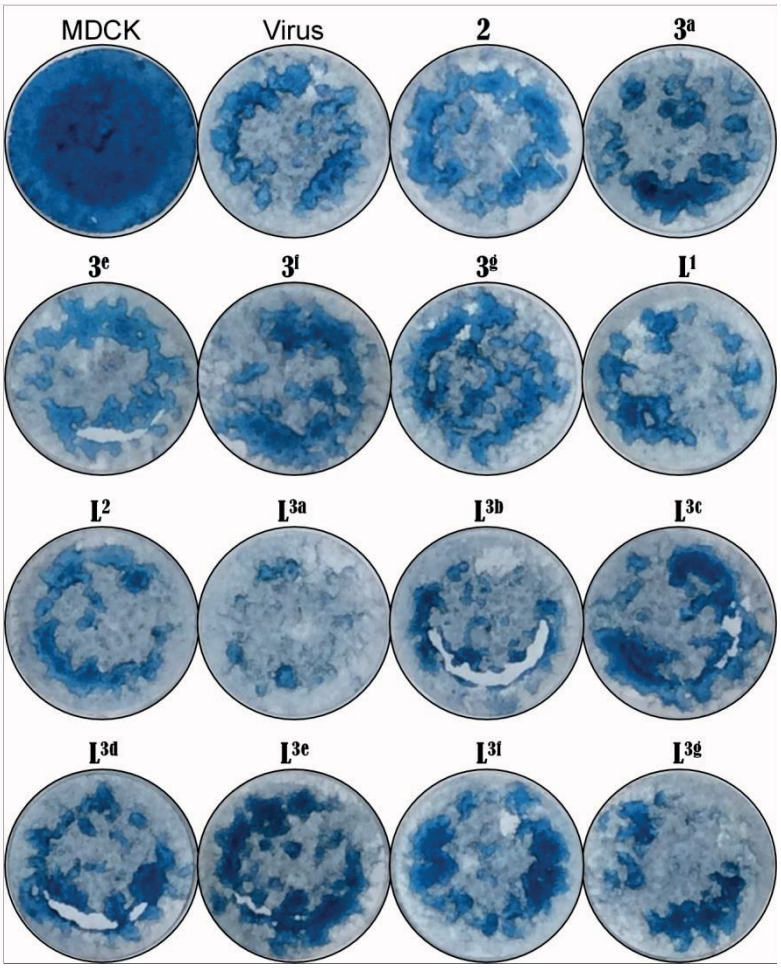


Figure 5. The effects of ligands and complexes on influenza A virus plaque formation. Plaque tests were carried out as described in Figure 4.

biological functionalities were investigated within the scope of the antiviral and cytotoxic activities. The compounds showed different levels of toxic effects on MDCK cells, with GI_{50} values between 15.9 and 161.8 μg/mL. The ligands did not show an antiviral effect against influenza viruses, but some of the complexes, ONS (**1**) and ONN (**3c** and **3d**), had considerable inhibitory effect on plaque formation of influenza A virus at lower concentrations than the GI_{50} values. The significant antiviral effect observed for the propyl and butyl substituted molecules gave rise to the thought that the average long alkyl chains activated the nickel-centered structure by optimizing the electron delocalization on the chelate system.

The experimental data did not lead to a systematic approach to the structure–activity relationship regarding these series of nickel complexes. However, the tests showed that a selective antiviral activity could be achieved through the choice of a coordinative donor atom and S-alkyl substituent. Additionally, the antiviral effects determined only in the form of the nickel(II)-PPh_3 complex independently from cytotoxicities of the compounds could be considered as a significant finding. Studies on the antiviral mechanisms of the compounds are in progress.

3. Experimental

3.1. General remarks

All chemicals were of reagent grade and used as commercially purchased without further purification. The elemental analyses were conducted on a Thermo Finnigan Flash EA 1112 Series Elemental Analyzer and Varian Spectra 220/FS atomic absorption spectrometer. IR spectra of the compounds were recorded with a Mattson 1000 FT-infrared spectrometer as KBr pellets. The ^{1}H and ^{31}P NMR spectra were recorded on a Bruker AVANCE-500 spectrometer.

3.2. Synthesis of the ligands

The thiosemicarbazones, $\mathbf{L^{1-3}}$, were prepared according to a previous study.[45] $\mathbf{L^{1}}$ and $\mathbf{L^{2}}$ are cream-colored and $\mathbf{L^{3a-3g}}$ (S-alkylated ligands) are yellow. The ligand structures were confirmed by means of elemental analysis and IR and ^{1}H NMR spectra. Aromatic and 3-methoxy protons of the thiosemicarbazones were in the region of 7.13–6.75 ppm and 3.84–3.86 ppm, respectively. Melting points (°C), yields (%), composition data, and some characteristic NMR peaks (ppm, $CDCl_3$) of the ligands are given below:

(2E)-2-[(2-Hydroxy-3-methoxyphenyl)methylidene]hydrazine-1-carbothioamide ($\mathbf{L^{1}}$): 241 °C; 95%. Anal. Calc. for $C_9H_{11}N_3O_2S$ (225.17 g): C, 47.99; H, 4.92; N, 18.65; S, 14.23. Found: C, 48.08; H, 4.99; N, 18.77; S, 14.35%. IR (KBr, cm^{-1}): ν(OH) 3338, ν(N^{4}H) 3307, 3115, ν(C-H) 2961, 2930, 2869, δ(N^{4}H) 1616, δ(N^{2}H) 1601, ν(C=N^1) 1546, ν(–C=S) 1220. NMR (500 MHz, $CDCl_3$): 11.05 (s, 1H, OH), 9.86 (s, 1H, N^{2}H), 8.58 (s, 1H, CH=N), 7.40 (s, 2H, N^{4}H), 7.13 (dd, J = 1.34, J = 1.46, 1H, d), 7.06 (d, J = 8.29, 1H, b), 6.91 (t, J = 7.81, 1H, c), 3.86 (s, 3H, O–CH_3).

(2E)-2-[(2-Hydroxy-3-methoxyphenyl)methylidene]-N-propylhydrazine-1-carbothioamide ($\mathbf{L^{2}}$): 221 °C; 90%. Anal. Calc. for $C_{12}H_{17}N_3O_2S$ (267.35 g): C, 53.91; H, 6.41; N, 15.72; S, 11.99. Found: C, 53.57; H, 6.16; N, 15.52; S, 11.79%. IR (KBr, cm^{-1}): ν(OH) 3461, ν(N^{4}H) 3346, ν(N^{2}H) 3153, δ (N^{4}H) 1618, δ (N^{2}H) 1605, ν(C=N^1) 1530, ν(–C=S) 1215. NMR (500 MHz, DMSO-d_6): 11.37 (s, 1H, OH), 9.17 (s, 2H, N^{2}H), 8.42 (t, 1H, N^{4}H), 8.39 (s, 1H, CH=N), 7.53 (d, J = 7.32, 1H, d), 6.96 (d, J = 6.37, 1H, b), 6.78 (t, J = 8.30, J = 7.81, 1H, c), 3.81(s, 3H, O–CH_3), 3.50 (q, 2H, N–CH_2), 1.59 (m, 2H, -CH_2-), 0.87 (t, J = 7.32, 3H, –CH_3).

Methyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate ($\mathbf{L^{3a}}$): 163 °C; 80%. Anal. Calc. for $C_{10}H_{13}N_3O_2S$ (239.29 g): C, 50.19; H, 5.48; N, 17.56; S, 13.40. Found: C, 50.26; H, 5.66; N, 17.65; S, 12.25. IR (KBr, cm^{-1}): ν(OH) 3400, ν(NH_2) 3307, 3130, ν(C-H) 2971, 2930, 2869, δ(N^{4}H) 1653, ν(C=N^1) 1600, ν(C=N^2) 1576. NMR (500 MHz, $CDCl_3$): 11.66 (s, 1H, OH), 8.40, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.84–6.78 (m, 1H, b, c, d), 5.05 (s, 2H, N^{4}H), 3.85 (s, 3H, O–CH_3), 2.44 (s, 3H, S–CH_3).

Ethyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate ($\mathbf{L^{3b}}$): 140 °C; 85%. Anal. Calc. for $C_{11}H_{15}N_3O_2S$ (253.32 g): C, 52.15; H, 5.97; N, 16.15; S, 12.66. Found: C, 52.23; H, 6.05; N, 16.19; S, 12.75%. IR (KBr, cm^{-1}): ν(OH) 3415, ν(NH_2) 3300, 3123, ν(C-H) 2960, 2928, 2864, δ(N^{4}H) 1646, ν(C=N^1) 1600, ν(C=N^2) 1576, ν(–C–S) 838. NMR (500 MHz, $CDCl_3$): 11.74 (s, 1H, OH), 8.38, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.85–6.77 (m, 3H, b, c, d), 5.08 (s, 2H, N^{4}H), 3.84 (s, 3H, O–CH_3), 3.02, 2.89 (q, i: 2/1, 2H, S–CH_2), 1.30 (t, 3H, –CH_3).

Propyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate ($\mathbf{L^{3c}}$): 267 °C; 87%.

Anal. Calc. for $C_{12}H_{17}N_3O_2S$ (267.35 g): C, 53.91; H, 6.41; N, 15.72; S, 11.99. Found: C, 53.95; H, 6.71; N, 15.72; S, 12.15%. IR (KBr, cm^{-1}): ν(OH) 3415, $\nu(N^4H)$ 3345, 3160, ν(C-H) 2961, 2930, 2869, $\delta(N^4H)$ 1653, $\nu(C{=}N^1)$ 1601, $\nu(C{=}N^2)$ 1576. NMR (500 MHz, $CDCl_3$): 11.68 (s, 1H, OH), 8.38, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.85–6.75 (m, 3H, b, c, d), 5.03 (s, 2H, N^4H), 3.85 (s, 3H, O–CH_3), 3.00, 2.87 (t, i: 2/1, J:7,32, J:6,83, 2H, S–CH_2), 1.73–1.65 (m, 2H, -CH_2-), 1.02–0.95 (m, 3H, –CH_3).

Butyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate (**L^{3d}**): 281 °C; 80%. Anal. Calc. for $C_{13}H_{19}N_3O_2S$ (281.37 g): C, 55.49; H, 6.81; N, 14.93; S, 11.40. Found: C, 55.56; H, 6.85; N, 14.98; S, 11.52%. IR (KBr, cm^{-1}): ν(OH) 3438, $\nu(N^4H)$ 3345, 3160, ν(C-H) 2953, 2923, 2830, $\delta(N^4H)$ 1650, $\nu(C{=}N^1)$ 1600, $\nu(C{=}N^2)$ 1576. NMR (500 MHz, $CDCl_3$): 11.67 (s, 1H, OH), 8.37, 8.24 (s, syn/anti: 2/1, 1H, CH=N), 6.86–6.77 (m, 3H, b, c, d), 5.04 (s, 2H, N^4H), 3.85 (s, 3H, O–CH_3), 3.02, 2.87 (t, i: 2/1, J:7,32, 2H, S–CH_2), 1.66–1.60, 1.42–1.36 (m, 4H, -CH_2-CH_2-), 0.87 (t, 3H, –CH_3).

Pentyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate (**L^{3e}**): 295 °C; 82%. Anal. Calc. for $C_{14}H_{21}N_3O_2S$ (295.40 g): C, 56.92; H, 7.17; N, 14.22; S, 10.86. Found: C, 56.99; H, 7.35; N, 14.30; S, 10.83%. IR (KBr, cm^{-1}): ν(OH) 3438, $\nu(N^4H)$ 3305, 3084, ν(C-H) 2961, 2930, 2869 $\delta(N^4H)$ 1653, $\nu(C{=}N^1)$ 1601, $\nu(C{=}N^2)$ 1576, ν(–C–S) 869. NMR (500 MHz, $CDCl_3$): 11.69, 11.49 (s, i: 2/1, 1H, OH), 8.37, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.86–6.77 (m, 3H, b, c, d), 5.02 (s, 2H, N^4H), 3.85 (s, 3H, O–CH_3), 3.01, 2.88 (t, i: 2/1, 2H, S–CH_2), 1.69–1.62 (m, 2H, -CH_2-), 1.37–1.28 (m, 4H, -CH_2-CH_2-), 0.84 (t, 3H, –CH_3).

Hexyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate (**L^{3f}**): 145 °C; 88%. Anal. Calc. for $C_{15}H_{23}N_3O_2S$ (309.43 g): C, 58.22; H, 7.49; N, 13.58; S, 10.36. Found: C, 58.36; H, 7.52; N, 13.58; S, 10.36%. IR (KBr, cm^{-1}): ν(OH) 3438, $\nu(N^4H)$ 3315, 3061, ν(C-H) 2946, 2923, 2853, $\delta(N^4H)$ 1660, $\nu(C{=}N^1)$ 1607, $\nu(C{=}N^2)$ 1561. NMR (500 MHz, $CDCl_3$): 11.69, 11.48 (s, i: 2/1, 1H, OH), 8.38, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.86–6.78 (m, 3H, b, c, d), 5.02 (s, 2H, N^4H), 3.85 (s, 3H, O–CH_3), 3.02, 2.88 (t, i: 2/1, 2H, S–CH_2), 1.67–1.61 (m, 2H, -CH_2-), 1.37–1.24 (m, 6H, (-CH_2-)$_3$), 0.84 (t, 3H, –CH_3).

Heptyl N'-[(E)-(2-hydroxy-3-methoxyphenyl)methylidene]carbamohydrazonothioate (**L^{3g}**): 148 °C; 84%. Anal. Calc. for $C_{16}H_{25}N_3O_2S$ (323.45 g): C, 59.41; H, 7.79; N, 12.99; S, 9.91. Found: C, 59.56; H, 7.85; N, 15.79; S, 13.15%. IR (KBr, cm^{-1}): ν(OH) 3576, $\nu(N^4H)$ 3345, 3160, ν(C-H) 2981, 2940, 2858, $\delta(N^4H)$ 1620, $\nu(C{=}N^1)$ 1601, $\nu(C{=}N^1)$ 1589, ν(–C–S) 869. NMR (500 MHz, $CDCl_3$): 11.70, 11.48 (s, i: 2/1, 1H, OH), 8.38, 8.25 (s, syn/anti: 2/1, 1H, CH=N), 6.86–6.78 (m, 3H, b, c, d), 5.02 (s, 2H, N^4H), 3.85 (s, 3H, O–CH_3), 3.01, 2.88 (t, i: 2/1, 2H, S–CH_2), 1.67–1.61 (m, 2H, -CH_2-), 1.38–1.34 (m, 2H, -CH_2-), 1.27–1.19 (m, 6H, (-CH_2-)$_3$), 0.82 (t, 3H, –CH_3).

3.3. Synthesis of the complexes

The nickel(II) complexes, **1**, **2**, and **3a**–**3g**, were synthesized with small modifications of the general procedures reported in the literature.[38,46] The solution of a thiosemicarbazone ligand (1 mmol) in dichloromethane (10 mL) was added dropwise to a solution of $[Ni(PPh_3)_2Cl_2]$ (1 mmol) in 10 mL of absolute ethanol. The mixture was stirred for 4 h at room temperature and left to stand for 1 week. The resulting product was filtered off and washed with 5 mL of n-hexane. The yield was calculated after drying in vacuo of the crystals obtained by recrystallization from 1:1 ethanol-dichloromethane.

The dark red complexes were identified by elemental analysis and IR, ^{1}H, and ^{31}P NMR spectra. IR spectra of **1**, **2**, and **3a**–**3g** included a $\nu(PPh_3)$ band at 1438, 1100, and 700 cm^{-1} in addition to the thiosemicarbazidato moieties. Signals of all aromatic protons were observed as doublets and triplets in the region of 7.86–6.50 ppm. Singlet peaks of the 3-methoxy protons were between 3.84 and 3.86 ppm. The melting points (°C), yields (%), and analytical and spectroscopic data showing the proposed structures are given.

1: 235 °C; 88%. Anal. Calc. for $C_{27}H_{24}N_3O_2PSNi$ (544.23 g): C, 59.59; H, 4.44; N, 7.72; S, 5.89. Found: C, 59.74; H, 4.62; N, 7.82; S, 6.05%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3453, $\delta(N^4H)$ 1623, $\nu(C{=}N^1)$ 1584, $\nu(C{=}N^2)$ 1538. ^{1}H NMR (500 MHz, $CDCl_3$): 8.17 (d, 1H, CH=N), 7.78–7.74 (m, 6H, p, t), 7.34–7.31 (m, 6H, q, s), 7.40 (dd, J = 1.95, J = 7.81, 3H, r), 6.85 (dd, J = 1.46, J = 7.81, 1H, d), 6.63 (dd, J = 1.96, J = 7.81, 1H, c), 6.46 (t, J = 7.81, 1H, b), 4.47 (s, 2H, N^4H). ^{31}P NMR: 29.56.

2: 167 °C; 85%. Anal. Calc. for $C_{30}H_{30}N_3O_2PSNi$ (586.31 g): C, 61.46; H, 5.28; N, 7.17; S, 5.47. Found: C, 61.50; H, 5.34; N, 7.22; S, 5.56%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3423, $\delta(N^4H)$ 1638, $\nu(C{=}N^1)$ 1600 $\nu(C{=}N^2)$ 1546. ^{1}H NMR (500 MHz, $CDCl_3$): 8.24 (s, 1H, CH=N), 7.86 (dd, J = 7.32, J = 10.73, 6H, p, t), 7.40 (t, J = 6.86, J = 7.81, 3H, r), 7.33 (t, J = 5.86, J = 7.81, 6H, q, s), 6.84 (d, J = 6.84, 1H, d), 6.61 (d, J = 6.84, 1H, b), 6.46 (t, J = 7.81, 1H, c), 3.63 (s, 1H, N^4H), 3.16–3.12 (q, 3H, N–CH_2), 1.49–1.42 (s, 1H, -CH_2-), 0.83 (t, 3H, –CH_3).

3a: 225 °C; 52%. Anal. Calc. for $C_{28}H_{27}N_3O_2PSNiCl$ (594.71 g): C, 56.55; H, 4.58; N, 7.07; S, 5.39. Found: C, 56.40; H, 4.65; N, 7.25; S, 5.68%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3384, $\delta(N^4H)$ 1638, $\nu(C{=}N^1)$ 1592, $\nu(C{=}N^2)$ 1576. ^{1}H NMR (500 MHz, $CDCl_3$): 8.43 (s, 1H, CH=N), 7.80–7.74 (m, 6H, p, t), 7.53 (t, J = 6.84, 3H, r), 7.48–7.40 (m, 6H, q, s), 6.94 (d, J = 7.81, 1H, d), 6.72 (d, J = 7.32, 1H, b), 6.61(d, J = 7.81, 1H, c), 3.65 (s, 1H, N^4H), 2.96 (t, 3H, S–CH_3).

3b: 129 °C; 70%. Anal. Calc. for $C_{29}H_{29}N_3O_2PSNiCl$ (608.74 g): C, 57.22; H, 4.80; N, 6.90; S, 5.27. Found: C, 57.10; H, 4.60; N, 7.05; S, 5.40%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3400, $\delta(N^4H)$ 1638, $\nu(C{=}N^1)$ 1607, $\nu(C{=}N^2)$ 1569. ^{1}H NMR (500 MHz, $CDCl_3$): 9.23 (s, 1H, CH=N), 7.79–6.72 (m, 15H, P-Ph), 6.69–6.63 (m, 3H, b, c, d), 3.99 (s, 1H, N^2H), 2.89 (m, 2H, S–CH_2), 1.39 (t, 3H, –CH_3).

3c: 204 °C; 75%. Anal. Calc. for $C_{30}H_{31}N_3O_2PSNiCl$ (622.77 g): C, 57.86; H, 5.03; N, 6.75; S, 5.15. Found: C, 57.70; H, 5.14; N, 6.85; S, 5.30%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3423, $\delta(N^4H)$ 1638, $\nu(C{=}N^1)$ 1600, $\nu(C{=}N^2)$ 1546. ^{1}H NMR: 8.43 (s, 1H, CH=N), 7.79–7.74 (m, 6H, p, t), 7.53 (t, J = 6.84, 3H, r), 7.48–7.40 (m, 6H, q, s), 6.94 (d, J = 7.81, 1H, d), 6.72 (d, J = 7.32, 1H, b), 6.61(d, J = 7.81, 1H, c), 5.20 (s, 1H, N^4H), 2.29 (t, 2H, S–CH_2), 1.20 (m, 2H, –CH_2-), 0.81 (t, 3H,–CH_3). ^{31}P NMR: 30.12.

3d: 209 °C; 80%. Anal. Calc. for $C_{31}H_{33}N_3O_2PSNiCl$ (636.79 g): C, 58.47; H, 5.22; N, 6.60; S, 5.04. Found: C, 57.29; H, 4.93; N, 6.93; S, 4.85%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3405, $\delta(N^4H)$ 1638, $\nu(C{=}N^1)$ 1615, $\nu(C{=}N^2)$ 1592. ^{1}H NMR (500 MHz, $CDCl_3$): 8.40 (s, 1H, CH=N), 7.72–7.58 (m, 6H, p, t), 7.50–7.46 (t, J = 6.35, 3H, r), 7.41–7.28 (m, 6H, q, s), 6.88 (d, J = 7.81, 1H, d), 6.66 (d, J = 7.32, 1H, b), 6.57 (t, J = 8.30, 1H, c), 5.23 (s, 1H, N^4H), 3.98, 3.66 (t, i:1, 2H, S–CH_2), 1.19–1.14 (m, 4H, -CH_2-CH_2-), 0.82 (t, 3H, –CH_3). ^{31}P NMR: 30.37.

3e: 146 °C; 75%. Anal. Calc. for $C_{32}H_{34}N_3O_2PSNi$ (614.36 g): C, 62.56; H, 5.58; N, 6.84; S, 5.22. Found: C, 61.37; H, 5.61; N, 6.46; S, 4.75%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3384, $\delta(N^4H)$ 1646, $\nu(C{=}N^1)$ 1600, $\nu(C{=}N^2)$ 1553. ^{1}H NMR (500 MHz, $CDCl_3$): 8.94 (s, 1H, CH=N), 7.86–7.40 (m, 15H, P-Ph), 7.03 (d,

J = 7.81, 1H, d), 6.67 (d, J = 6.83, 1H, b), 6.58 (d, J = 6.84, 1H, c), 3.65 (s, 1H, N^4H), 2.69 (t, 2H, S–CH_2), 1.42–1.35 (m, 2H, -CH_2-), 1.20–1.14 (m, 4H, -CH_2-CH_2-), 0.79 (t, 3H, –CH_3).

3f: 132 °C; 75%. Anal. Calc. for $C_{33}H_{36}N_3O_2PSNi$ (628.39 g): C, 63.07; H, 5.77; N, 6.69; S, 5.10. Found: C, 62.83; H, 6.03; N, 6.49; S, 4.88%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3384, $\delta(N^4H)$ 1653, $\nu(C{=}N^1)$ 1600, $\nu(C{=}N^2)$ 1553. ^{1}H NMR (500 MHz, $CDCl_3$): 8.36 (s, 1H, CH=N), 9.61 (s, 1H, N^2H), 7.78–7.19 (m, 15H, P-Ph), 6.93–6.60 (m, 3H, b, c, d), 3.86 (t, 2H, S–CH_2), 1.80–1.14 (m, 8H, (-CH_2-)$_4$), 0.81 (t, 3H, –CH_3).

3g: 87 °C; 75%. Anal. Calc. for $C_{34}H_{38}N_3O_2PSNi$ (642.42 g): C, 63.57; H, 5.96; N, 6.54; S, 4.99. Found: C, 63.36; H, 6.14; N, 6.80; S, 4.61%. IR (KBr, cm^{-1}): $\nu(N^4H)$ 3423, $\delta(N^4H)$ 1630, $\nu(C{=}N^1)$ 1600, $\nu(C{=}N^2)$ 1553. ^{1}H NMR (500 MHz, $CDCl_3$): 8.25 (s, 1H, CH=N), 9.20 (s, 1H, N^2H), 7.76–6.50 (m, 18H, aromatic), 2.78 (t, 2H, S–CH_2), 1.49–1.18 (m, 10H, (-CH_2-)$_5$), 0.81 (t, 3H, –CH_3).

3.4. Cells and viruses

Madin–Darby canine kidney (MDCK) cells were used as host cells.[47,48] These cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (GIBCO), penicillin G (100 U/mL), and streptomycin (100 mg/mL). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Influenza virus strain A/WSN/33 (H1N1) was kindly provided by Dr Kyosuke Nagata of the Faculty of Medicine, University of Tsukuba, Tsukuba, Japan. The viruses were grown in the allantoic cavity of 10-day-old chick embryos at 35.5 °C for 48 h. The allantoic fluid was harvested and stored in small aliquots at –80 °C.

3.5. Plaque assay

Confluent MDCK cells in 12-well plates were washed twice with DMEM and infected with influenza A viruses at the appropriate multiplicity of infection. After adsorption for 30 min at 37 °C, the infecting medium was completely removed and the cell monolayers were overlaid with maintenance medium (DMEM containing 0.6% agarose, 0.2% bovine serum albumin, and 4 μg/mL trypsin).[49] In test conditions, nickel(II)-PPh_3 compounds were added to the maintenance medium at defined concentrations. The cultures were incubated at 34 °C for 3 days and plaques were visualized by staining cells with Amido black.

3.6. Determination of cytotoxicity

To determine the cytotoxic concentration inhibiting of 50% of cell growth (GI_{50}), confluent MDCK cell monolayers grown in 96-well plates were incubated with serial twofold dilutions of compounds for 48 h in a humidified atmosphere containing 5% CO_2 at 37 °C. Then the cell viability was detected by MTT assay.[50] Growth media were removed and 100 μ L of MTT solution (0.5 mg/mL in PBS) was added to each well. The plates were then further incubated for 4 h at 37 ° C. Thereafter, insoluble formazan crystals were solubilized with acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol) and absorbance was measured at 570 nm by ELISA reader (Epoch Microplate Spectrophotometer, BioTek, USA).

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