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ORIGINAL RESEARCH

Synthesis and antiviral activity of some pyrrolonyl substituted heterocycles as additives to enhance inactivated Newcastle disease vaccine

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Abstract

This research reported the design and synthesis of some influential new pyrrolone derivatives bearing a pyrazole scaffold with the evaluation of their antiviral activity against Newcastle disease virus (NDV) in specific pathogen free (SPF) chicken embryos and immune boosting properties of these substances in SPF chicks. The building block synthon was the pyrazolyl acid hydrazide, derived from 2(3*H*)-furanone, which was reacted with some carbonyl compounds, e.g., salicylaldehyde, furfural, 1,3-diphenylpyrazole-4-carbaldehyde, 2-chloroquinoline-3-carbaldehyde, chromone-3-carbaldehyde, and 3-acetylcoumarin. The results revealed that pyrazole derivative **6**, quinoline derivatives **7** and **8** exhibited 100% protection against NDV while the hydroxyphenyl derivative **3** showed 95% protection. In turn, chromone derivative **10** and coumarin derivative **11** exhibited 90% protection. The structures of all products were established on the basis of their elemental analyses and spectroscopic techniques.

Keywords Newcastle disease · SPF chicks · Pyrazole · Acid hydrazide · Chromone

Introduction

Infectious diseases are one of the factors responsible for decreased in the production of poultry products. Poultry flocks are encountered with Newcastle disease, and are responsible for high rate of economic losses (Qureshi et al. 1981; Siddique et al. 2012; Numan et al. 2005; Cheema et al. 2011). Newcastle disease virus (NDV) is a widespread and economically important poultry pathogen. Based on their pathogenicity for chickens, NDV strains are classified into highly pathogenic (velogenic), intermediate (mesogenic), and apathogenic (lentogenic) strains (Römer-Oberdörfer et al. 2003; Aamir et al. 2014; Lee 2009; Miller 2016). Considerable research efforts have been devoted to the detection of new antiviral natural products (Mbanga et al. 2010; Perera and Efferth 2012; Watanabe et al. 2014). Noteworthy, the pyrazole compounds are a ubiquitous feature of a pharmacophoric scaffold, which represents a class of *N*-heterocyclic compounds with a wide range of biological and pharmacological applications. Many of them are widely used as potent antiviral against HPAI H5N1 influenza virus, hepatitis A virus, herpes simplex virus type 1, anti-HIV, hepatitis C virus, and hypoxia inducible factor (Ramadan and Abou-Elmagd 2018; Abou-Elmagd et al. 2016; Shih et al. 2010; El-Sabbagh et al. 2009; Rashad et al. 2008; Hashem et al. 2007; Ouyang et al. 2008; Riyadh et al. 2010; Perez-Fernandez et al. 2014), anticancer (Riyadh et al. 2010; Perez-Fernandez et al. 2014; Abou-Elmagd et al. 2016; Ramadan and Sallam 2018; Ghadbeigi et al. 2015). Also, it was reported that BPR1P0034 has potent inhibitory activity against influenza virus. BPR1P0034 was the first pyrazole-based anti-influenza compound (Shih et al. 2010), as well as, some FDA approved drugs based on the pyrazole ring like ruxolitinib, crizotinib, or tozasertib (Fig. 1)

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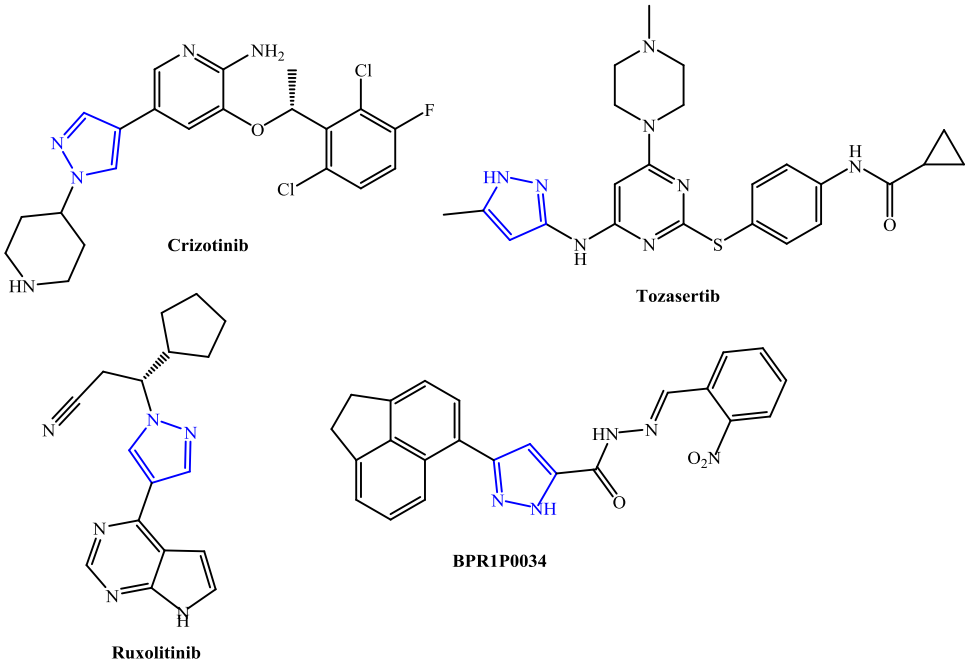


Fig. 1 Some FDA approved drugs based on the pyrazole ring

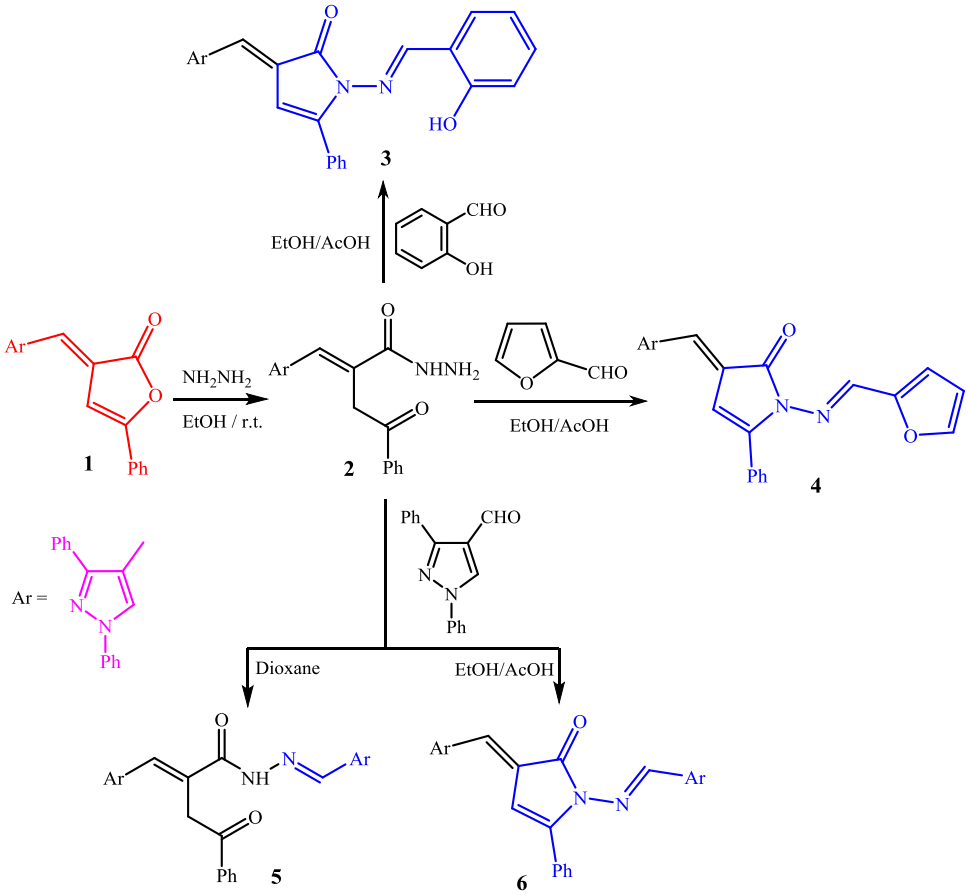
(Nitulescu et al. 2019, 2015; Keter and Darkwa 2012). Herein, we report an approach to explore and expand the antiviral activity of synthesized series of pyrazole derivatives, via the structural modifications. Our rational design was founded on structural diversification through conserving the pyrazole scaffold with different hydrazone and pyrrolone moieties, to attain an active antiviral agent with an improved activity and selectivity toward NDV in SPF chicken embryos and evaluation of immune boosting properties of these substances in SPF chicks.

Results and discussion

Chemistry

The readily obtainable acid hydrazide **2**, derived from the corresponding 2(3*H*)-furanone **1** (Hashem et al. 2007), toward different carbonyl compounds was successfully investigated. Indeed, condensation with salicyladehyde and furfural in absolute ethanol containing few drops of glacial acetic acid prompted the construction of pyrrolone **3** and **4** in 80% and 76% yields, respectively (Scheme 1). The structures of compounds **3** and **4** were substantiated from their analytical and spectroscopic data. Thus, the IR spectra were devoid absorption bands of NH_2 group. The ^{1}H NMR spectra fitted with the assigned structures. In turn, the reaction of the hydrazide **2** with 1,3-diphenylpyrazole-4-carboxaldehyde was mainly dependent on the reaction media in an attempt to synthesize pyrrolone derivative bearing two pyrazolyl moiety used for the investigation of its antiviral activity. Thus, when the mentioned aldehyde was treated with hydrazide **2** in refluxing dioxane, the corresponding hydrazone **5** was obtained. Investigation of the reaction in refluxing EtOH/AcOH led to ring closure to furnish the pyrrolone derivative **6** (Scheme 1). The ^{1}H NMR spectrum of hydrazone **5** provided singlet for methylene protons, as well as, the NH singlet exchangeable with D_2O. The plausible pathway for the formation of the pyrrolone **6** could be illustrated via 5-exo-trig cyclization of the corresponding hydrazone **5**.

In turn, focusing on reactivity of the hydrazide moiety toward 2-chloroquinoline-3-carboxaldehyde and chromone-3-carboxaldehyde led us to explore the utilization of the hydrazide derivative **2** as a precursor to construct more heterocycles. Thus, treatment of the hydrazide **2** with the former aldehyde in refluxing dioxane afforded the hydrazone derivative **7**, while in refluxing EtOH/AcOH, the pyrrolone **8** was obtained in good yield (Scheme 2). The IR spectrum of quinolinyl hydrazone **7** exhibited the characteristic stretching absorption bands for OH, NH, C=O and C=N groups at ν 3422, 3161, 1669, and 1612 cm^{-1}, respectively. The ^{1}H NMR spectra were in accordance with assigned structures. A compelling evidence for the structure of compounds **7** and **8** were gained from their ^{13}C NMR spectra, which provided the characteristic signals to these structures. Furthermore, their mass spectra exhibited the correct molecular ion peak and some important abundant peaks (cf. "Experimental"). Formation of compound **7** could be visualized to occur *via* elimination of water molecule followed by removal of chlorine under heating conditions to produce



Scheme 1 Condensation of hydrazide **2** with salicylaldehyde, furfural and 1,3-diphenylpyrazole-4-carboxaldehyde

hydroxyquinoline derivative **7**. The formation of pyrrolone **8** was explained via cyclization of compound **7**.

When the hydrazide **2** was reacted with chromone-3-carboxaldehyde in boiling dioxane, the corresponding hydrazone **9** was formed (Scheme 2). Pyrrolone derivative **10** was obtained when the reaction was carried out in refluxing EtOH/AcOH which prompted the cyclization. The spectral data were well correlated with the assigned structures (cf. "Experimental"). On the other hand, condensation of the hydrazide **2** with 3-acetylcoumarin in boiling dioxane was smoothly achieved to afford the corresponding hydrazone derivative **11** (Scheme 2).

Biological activity studies

Antiviral activity

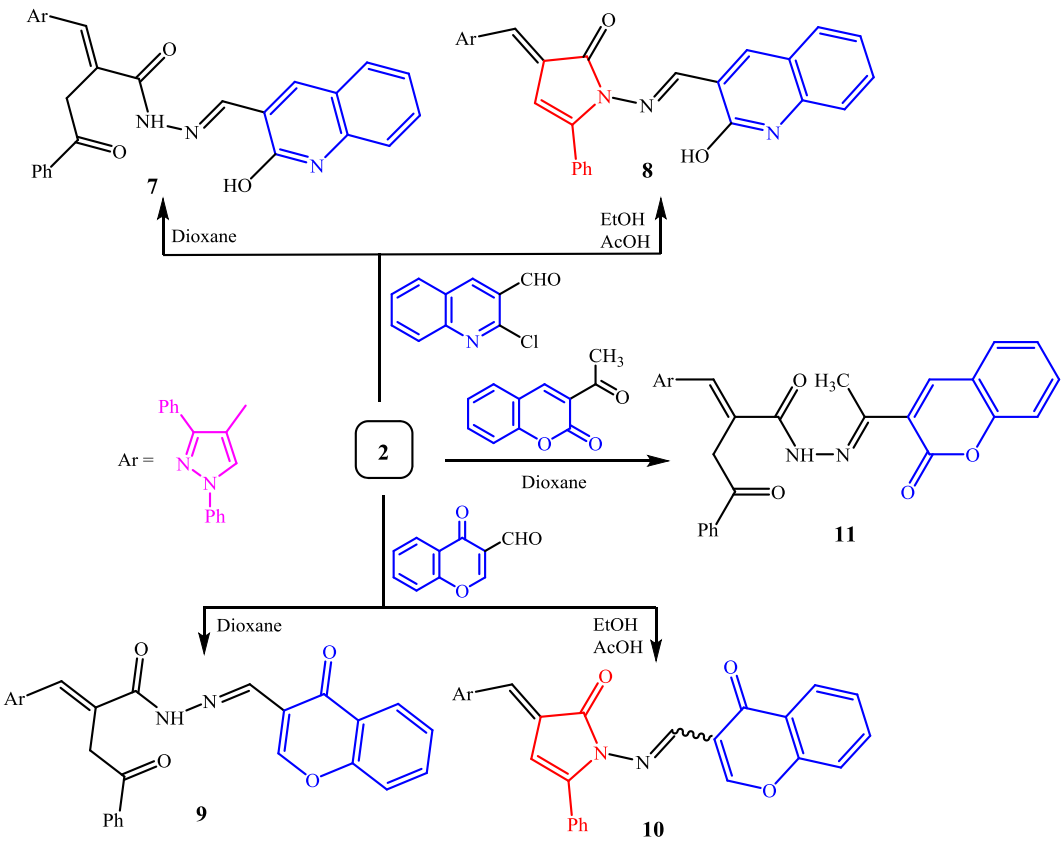
Haemagglutination is the aggregation of red blood cells (RBCs) in suspension with the presence of certain (haemagglutinating) virus particles. This phenomenon is a result of the attachment of specific outer viral peplomeres (haemagglutinins) with specific receptors present on the surface of RBCs. This characteristic feature can be used in detection of the virus. The inhibition of haemagglutination caused by these viruses is the detection of their activity's inhibition. Ribavirin was used as standard reference drug.

In each case of the tested compounds, the toxicity assays in embryonated chicken SPF eggs indicated that at concentration (CC_{50}) ranging from 200 to 800 µL/egg (Table 1). The therapeutic index (TI) of the tested compound was expressed as CC_{50}/IC_{50} was calculated using the method described (Reed and Muench 1938).

The six compounds showed promising antiviral activity. Embryonated eggs inoculated with a mixture of NDV and each compound separately affected by the virus replication (12-fold serial dilutions of the mixtures were performed), with the allantoic fluid from each group of eggs receiving varying serial dilutions of the mixtures. This result showed that virus titer was affected (Table 2).

Bioassay of immune boosting properties of different compounds in SPF chicks

Blood samples were collected individually from jugular vein at 28 days post vaccination (dpv) for potency test by calculation of the HI antibody titer in serum of vaccinated chicks. Comparison of humeral response of the vaccinated group (first group vaccinated only not receive any



Scheme 2 Synthesis of quinoline, chromone and coumarin derivatives **7–11**

Table 1 The different IC_{50} in embryonated chicken SPF eggs and their embryonic toxicity (NDV)

Compound	CC_{50}	IC_{50}	TI (%)
3	>800	≤9	88.8
6	>400	≤4	100
7	>200	≤2	100
8	>700	≤7	100
10	>300	≤6	50
11	>500	≤8	62.5
Ribavirin	>200	≤2	100

NDV Newcastle disease virus, *CC_{50}* cytotoxicity concentration fifty, *IC_{50}* the antiviral inhibitory concentration fifty, *TI* the therapeutic index

substances) and other vaccinated groups which separately received the tested compounds as immunostimulant revealed that compound **7** has special immune boosting properties as it elevates the antibody titer in the serum of vaccinated chicks (Table 3). Protection percent of the vaccine was calculated as the number of live birds/number of dead birds × 100. The results revealed that pyrazole derivative **6**, quinoline derivatives **7**, and **8** exhibited 100% protection against NDV while the hydroxyphenyl derivative **3** showed 95% protection. In turn, chromone derivative **10** and coumarin derivative **11** exhibited 90% protection.

The HI titer was 6.7 log2 (compound **3**)
The HI titer was 6.9 log2 (compound **6**)
The HI titer was 7.1 log2 (compound **7**)
The HI titer was 7 log2 (compound **8**)
The HI titer was 6.4 log2 (compound **10**)
The HI titer was 6.6 log2 (compound **11**)
The HI titer was 7.1 log2 (Ribavirin as standard compound).
While it was 6.6 log2 (vaccinated group only).

Structure-activity relationship (SAR)

Incorporation of 2-hydroxybenzylidene (as in compound **3**) enhanced the protection percent (95%). Introduction of an additional pyrazole moiety (as in compound **6**) or quinoline moieties (as in the hydrazone **7** and pyrrolone **8**) linked to the parent pyrazole scaffold resulted in remarkable potency (100%) as compared with the rest of compounds (Fig. 2). However, chromone and coumarin moieties (as in compounds **10** and **11**) might have a relative positive impact on their activities with respect to other derivatives.

Conclusion

Design and synthesis of some *N*-heterocycles bearing a pyrazole scaffold were achieved and later screened for their antiviral activity against NDV in SPF chicken embryos and evaluation of immune boosting properties of these substances in SPF chicks. Comparison of humeral response of the vaccinated group (first group vaccinated only not receive any substances) and other vaccinated groups which separately received the tested compounds as immunostimulant revealed that the quinoline derivative has special immune boosting properties as it elevates the antibody titer in the serum of vaccinated chicks.

Experimental

Chemistry

General

Melting points were measured on a GALLENKAMP electric melting point apparatus (SANYO GALLENKAMP, UK), and are uncorrected. The reactions were monitored by thin-layer chromatography using precoated Merck Kiesel gel 60 F_{254} aluminum backed plates obtained from Fluka, Switzerland. All reagents and solvents were purified and dried by standard techniques. The infrared spectra were recorded using potassium bromide disks on FTIR Thermo Electron Nicolet iS10 (USA) infrared spectrometer and expressed in wave number (ν, cm^{-1}). The ^1H NMR spectra were run at 400 MHz on a GEMINI NMR spectrometer using tetramethyl silane (TMS) as internal standard in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts (δ) are quoted in ppm. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. All coupling constant (J) values are given in hertz. The ^{13}C NMR spectra were run at 100 MHz on BRUKER NMR spectrometer (BRUKER, Manufacturing & Engineering Inc., Anaheim, CA, USA) using TMS as internal standard in DMSO. Mass spectrum was carried out on direct probe controller inlet part to single quadrupole mass analyzer in Thermo Scientific GCMS, MODEL ISQLT operating at 70 eV using Thermo X-CALIBUR software. The antiviral activity was performed against NDV in specific pathogen free (SPF) chicken embryos and immune boosting properties of these substances in SPF chicks. Ribavirin was used as standard reference drug. The starting hydrazide derivative **2** was previously synthesized according to the reported procedure (Hashem et al. 2007).

Condensation of the hydrazide 2 with salicylaldehyde and furfural

A solution of the hydrazide **2** (2.11 g, 5 mmol) and salicylaldehyde (0.61 g, 5 mmol) or furan-2-carbaldehyde (0.48 g, 5 mmol) in absolute ethanol (20 mL) containing three drops of glacial acetic acid was heated under reflux for 1 h. The precipitated solid while hot was then collected by filtration and recrystallized from ethanol/dioxane mixture (3:1) to afford the pyrrolone derivative **3** and **4**, respectively.

3-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-1-((-2-hydroxybenzylidene)amino)-5-phenyl-1,3-dihydro-2H-pyrrol-2-one (3) Orange crystals, mp. 221–223 °C, yield 80%. IR (KBr, ν, cm^{-1}): 3443 (OH), 3064 (CH-aromatic), 1684 (C=O), 1618 (C=N). ^1H NMR (400 MHz, DMSO-d_6, δ, ppm): 10.02 (s, 1H, OH, exchangeable), 9.19 (s, 1H, C_5–H pyrazole), 9.08 (s, 1H, CH=N), 7.82–7.36 (m, 15H,

Table 2 Haemagglutination activity of NDV incubated alone or separately with different types of compounds in embryonated chicken eggs (ECE_S)

		NDV alone	**3**	**6**	**7**	**8**	**10**	**11**	Ribavirin
Virus dilution	10^1	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	10^2	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	10^3	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	10^4	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	10^5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	10^6	5/5	4/5	5/5	4/5	5/5	4/5	4/5	4/5
	10^7	4/5	4/5	5/5	4/5	5/5	4/5	4/5	4/5
	10^8	3/5	3/5	5/5	3/5	5/5	3/5	3/5	3/5
	10^9	2/5	2/5	3/5	2/5	2/5	2/5	2/5	2/5
	10^{10}	1/5	0/5	2/5	0/5	1/5	0/5	0/5	0/5
	10^{11}	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
	10^{12}	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
HA-titer		10^{10}	10^9	$10^{8.6}$	$10^{8.6}$	$10^{8.6}$	$10^{9.8}$	$10^{9.6}$	$10^{8.6}$

HA Haemagglutination

Phenyl), 7.21 (s, 1H, CH=), 7.19–7.02 (m, 4H, Ar–H), 6.53 (s, 1H, C_4–H pyrrole). EIMS (70 eV, *m/z*, %): 506.19 ([M^+-2], 5), 504.20 (100), 476.19 (7), 460.14 (8), 423.27 (5), 359.48 (5), 251.06 (9), 278.18 (10), 172.26 (4), 108.94 (6), 77.18 (64). Anal. Calcd. for $C_{33}H_{24}N_4O_2$ (508.58): C, 77.94; H, 4.76; N, 11.02. Found: C, 77.82; H, 4.59; N, 11.01%.

3-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-1-((furan-2-ylmethylene)amino)-5-phenyl-1,3-dihydro-2H-pyrrol-2-one (4) Orange crystals, mp. 268–270 °C, yield 76%. IR (KBr, ν, cm^{-1}): 3055 (CH-aromatic), 1693 (C=O pyrrolone), 1621 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.33 (s, 1H, CH=N), 8.89 (s, 1H, C_5–H pyrazole), 8.09–8.06 (d, 1H, C_5–H furan, $J = 7.5$ Hz), 7.96–7.94 (d, 2H, Ar–H, $J =$ 8.1 Hz), 7.84–7.82 (d, 2H, Ar–H, $J = 7.2$ Hz), 7.74–7.73 (d, 2H, Ar–H, $J = 7.00$ Hz), 7.66–7.53 (m, 9H, Ar–H), 7.51–7.44 (m, 2H, furan), 7.23 (s, 1H, CH=), 6.99 (s, 1H, C_4-H pyrrole). EIMS (70 eV, *m/z*, %): 482.09 (M^+, 26), 460.82 (53), 441.26 (40), 384.33 (40), 308.94 (30), 280.30 (43), 223.11 (40), 150.50 (35), 83.35 (100), 72.04 (46). Anal. Calcd. for $C_{31}H_{22}N_4O_2$ (482.54): C, 77.16; H, 4.60; N, 11.61. Found: C, 77.01; H, 4.45; N, 11.57%.

Condensation of the hydrazide 2 with 1,3-diphenylpyrazole-4-carboxaldehyde

A mixture of the hydrazide **2** (2.11 g, 5 mmol) and 1,3-diphenylpyrazole-4-carboxaldehyde (1.24 g, 5 mmol) in dioxane was heated under reflux for 3 h. The reaction mixture was evaporated under vacuum. The residue was triturated with ether, filtered off and then recrystallized from ethanol/dioxane mixture (1:1) to produce the hydrazone derivative **5**. When the reaction was carried out in refluxing ethanol/glacial acetic acid, the precipitated solid while hot was filtered off and recrystallized from ethanol/dioxane mixture (2:1) to furnish the pyrrolone derivative **6**.

Table 3 Protection percent against NDV challenge virus

Vaccinated groups (20 chicks)	1st DPC	2nd DPC	3rd DPC	4th DPC	5th DPC	6th DPC	Protection %
3	0/20	0/20	0/20	0/20	1/20	0/20	95
6	0/20	0/20	0/20	0/20	0/20	0/20	100
7	0/20	0/20	0/20	0/20	0/20	0/20	100
8	0/20	0/20	0/20	0/20	0/20	0/20	100
10	0/20	0/20	0/20	1/20	0/20	1/20	90
11	0/20	1/20	0/20	1/20	0/20	0/20	90
Vaccinated group only	0/20	0/20	0/20	1/20	0/20	1/20	90
Control chicks	0/20	0/20	5/20	6/20	6/20	3/20	0
Ribavirin	0/20	0/20	0/20	0/20	0/20	0/20	100

Dead birds/live birds

DPC day post challenge

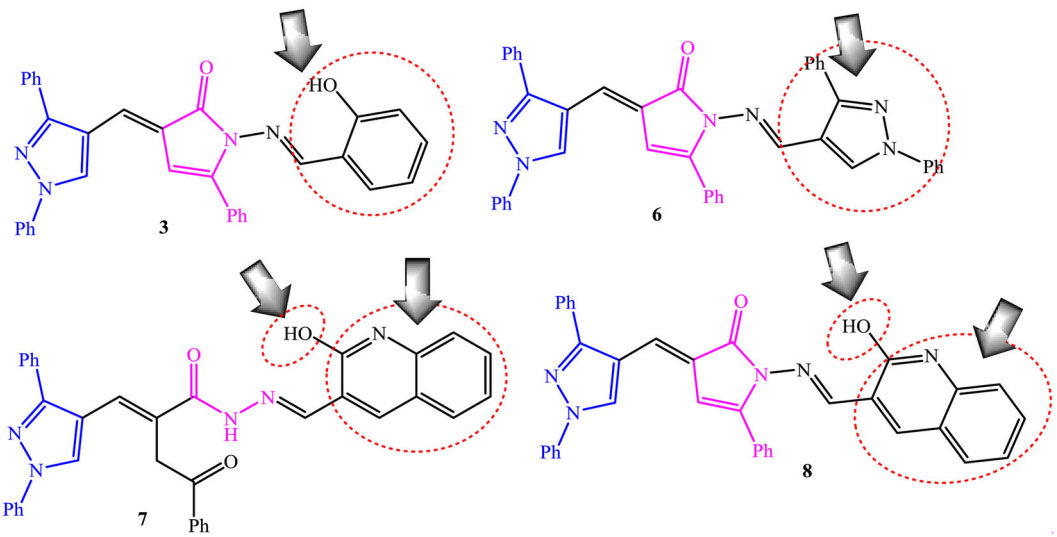


Fig. 2 SAR of the potent compounds

Synthesis of 2-((1,3-diphenyl-1H-pyrazol-4-yl)methylene)-N′-(-(1,3-diphenyl-1H-pyrazol-4-yl)methylene)-4-oxo-4-phenylbutanehydrazide (5) Yellow crystals, mp. 282–284 °C, yield 73%. IR (KBr, ν, cm^{-1}): 3059 (CH-aromatic), 2935 (CH-aliphatic), 1695 (C=O ketone), 1661 (C=O amide), 1620 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.06 (s, 1H, NH, exchangeable), 9.33 (s, 1H, CH=N), 9.26 (s, 1H, C_5–H pyrazole), 8.90 (s, 1H, C_5-H pyrazole), 8.08–7.35 (m, 25H, Ph-H), 7.23 (s, 1H, CH=), 3.54 (s, 2H, CH_2). Anal. Calcd. for $C_{42}H_{32}N_6O_2$ (652.76): C, 77.28; H, 4.94; N, 12.87. Found: C, 77.19; H, 4.81; N, 12.82%.

3-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-1-(((1,3-diphenyl-1H-pyrazol-4-yl)methylene)amino)-5-phenyl-1,3-dihydro-2H-pyrrol-2-one (6) Orange crystals, mp. 270–272 °C, yield 84%. IR (KBr, ν, cm^{-1}): 3059 (CH-aromatic), 1688 (C=O), 1658 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.33 (s, 1H, CH=N), 9.25 (s, 1H, C_5–H pyrazole), 8.88 (s, 1H, C_5–H pyrazole), 8.07–7.34 (m, 25H, Ar–H), 7.22 (s, 1H, CH=), 6.99 (s, 1H, C_4–H pyrrole). ^{13}C NMR (100 MHz, DMSO, δ, ppm): 90.51, 116.97, 117.09, 117.57, 119.42, 119.48, 119.84, 121.34, 125.48, 126.80, 127.39, 127.50, 128.36, 128.67, 128.74, 128.91, 129.17, 129.22, 129.34, 129.89, 130.04, 132.14, 132.54, 139.43, 139.54, 143.85, 144.26, 152.17, 153.38 (C=N), 164.70 (C=O). EIMS (70 eV, *m/z*, %): 634.09 (M^+, 28.65), 576.72 (22.92), 527.04 (74.66), 470.18 (17.95), 466.45 (25.74), 433.07 (29.66), 425.18 (62.22), 409.34 (35.08), 408.00 (100.00), 405.75 (34.38), 394.53 (33.64), 384.07 (35.54), 364.44 (24.42), 353.60 (31.62), 347.07 (30.48), 338.77 (33.52), 305.41 (29.50), 289.34 (28.55), 232.87 (42.65), 230.75 (31.77), 194.71 (32.60), 187.13 (66.64), 170.22 (31.50), 130.10 (14.30), 85.15 (19.55), 64.58 (34.47). Anal. Calcd. for $C_{42}H_{30}N_6O$ (634.74): C, 79.48; H, 4.76; N, 13.24. Found: C, 79.32; H, 4.61; N, 13.26%.

Condensation of the hydrazide 2 with 2-chloroquinoline-3-carboxaldehyde

A solution of the hydrazide **2** (2.11 g, 5 mmol), 2-chloroquinoline-3-carboxaldehyde (0.95 g, 5 mmol) in dioxane (20 mL) was heated under reflux for 30 min. The precipitated solid while heating was filtered off and recrystallized from dioxane to produce the quinoline derivative **7**. When the reaction mixture was heated under reflux in absolute ethanol (20 mL) containing three drops of glacial acetic acid for 1 h, the precipitated solid while hot was filtered off and recrystallized from ethanol/dioxane mixture (2:1) to afford the pyrrolone derivative **8**.

2-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-N′-((2-hydroxyquinolin-3-yl)methylene)-4-oxo-4-phenylbutanehydrazide (7) Yellow crystals, mp. 330–332 °C, yield 77%. IR (KBr, ν, cm^{-1}): 3422 (OH), 3161 (NH), 3059, 3007 (CH-aromatic), 2954, 2895 (CH-aliphatic), 1669 (C=O), 1612 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.15 (s, 2H, OH + NH, exchangeable), 9.14 (s, 1H, CH=N), 8.90 (s, 1H, C_5–H pyrazole), 8.83 (s, 1H, C_4–H quinoline), 8.24–8.22 (d, 1H, C_5–H quinoline, $J = 8$ Hz), 8.00–7.98 (m, 3H, quinoline-H), 7.87–7.36 (m, 15H, Aryl-H), 7.22 (s, 1H, CH=), 3.54 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO, δ, ppm): 66.82 (CH_2), 102.97, 104.53, 105.75, 107.48, 109.04, 110.60, 111.81, 112.51, 115.98, 118.92, 120.66, 123.26, 125.16, 126.72, 127.76, 130.19, 132.44, 133.14, 135.91, 137.99, 140.59, 142.50, 145.45, 147.70, 149.61 (C=N), 167.81 (N–C=O), 177.69 (N=C–OH), 196.76 (C=O ketone). EIMS (70 eV, *m/z*, %): 577.04 (M^+, 26.93), 541.32 (40.14), 507.46 (54.70), 483.74 (44.54), 441.67 (43.34), 411.03 (61.81), 395.83 (51.65), 376.67 (43.89), 362.02 (71.87), 348.17 (37.34), 330.01 (52.15), 308.73 (75.58), 301.27 (56.86), 217.16 (51.15), 201.28 (32.73), 177.89 (53.35), 159.40 (53.10), 140.81 (40.89), 137.26 (100.00), 130.66 (95.25), 126.75 (51.75), 113.68 (38.19), 91.72 (63.36), 74.51 (59.51). Anal. Calcd. for $C_{36}H_{27}N_5O_3$ (577.64): C, 74.86; H, 4.71; N, 12.12. Found: C, 74.71; H, 4.60; N, 12.09%.

3-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-1-(((2-hydroxyquinolin-3-yl)methylene)amino)-5-phenyl-1,3-dihydro-2H-pyrrol-2-one (8) Orange crystals, mp. 237–239 °C, yield 80%. IR (KBr, ν, cm^{-1}): 3442 (OH), 3058 (CH-aromatic), 1699 (C=O), 1617 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.13 (s, 1H, OH, exchangeable), 9.33 (s, 1H, CH=N), 9.25 (s, 1H, C_5-H pyrazole), 8.89 (s, 1H, C_4–H quinoline), 8.08–8.06 (d, 1H, C_5–H quinoline, $J =$ 8 Hz), 7.96–7.82 (m, 3H, quinoline-H), 7.75–7.38 (m, 15H, Aryl-H), 7.23 (s, 1H, CH=), 7.00 (s, 1H, C_4–H pyrrole). ^{13}C NMR (100 MHz, DMSO, δ, ppm): EIMS (70 eV, *m/z*, %): 559.91 (M^+, 49.94), 556.79 (28.44), 548.86 (32.66), 544.26 (48.26), 541.51 (61.02), 534.86 (41.66), 512.23 (33.42), 500.07 (28.63), 486.94 (66.73), 481.86 (67.08), 474.31 (73.97), 469.29 (55.88), 459.23 (55.88), 452.99 (66.46), 447.17 (54.70), 436.17 (98.43), 416.88 (100.00), 406.74 (69.07), 391.52 (31.81), 378.58 (50.90), 367.16 (37.98), 365.82 (40.78), 353.80 (56.42), 347.06 (49.75), 330.68 (35.30), 322.21 (28.36). Anal. Calcd. for $C_{36}H_{25}N_5O_2$ (559.63): C, 77.26; H, 4.50; N, 12.51. Found: C, 77.14; H, 4.38; N, 12.53%.

Condensation of the hydrazide 2 with 4-oxo-4*H*-chromene-3-carbaldehyde

A solution of the hydrazide **2** (2.11 g, 5 mmol) and 4-oxo-4*H*-chromene-3-carbaldehyde (0.87 g, 5 mmol) in dioxane (15 mL) was heated under reflux for 1 h. The separated solid while hot was collected by filtration and then

recrystallized from dioxane to give the corresponding hydrazone derivative **9**. When the reaction mixture was heated under reflux in absolute ethanol (20 mL) containing three drops of glacial acetic acid for 30 min, the precipitated solid while hot was collected by filtration and recrystallized from ethanol/dioxane mixture (2:1) to produce the pyrrolone derivative **10**.

2-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-4-oxo-N′-((4-oxo-4H-chromen-3-yl)methylene)-4-phenylbutanehydrazide (9) Yellow crystals, mp. 256–258 °C, yield 79%. IR (KBr, ν, cm^{-1}): 3278 (NH), 3057 (CH-aromatic), 2955, 2916 (CH-aliphatic), 1694 (C=O ketone), 1663 (C=O chromone and amide), 1617 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.03 (s, 1H, NH, exchangeable), 8.76 (s, 1H, CH=N), 8.63 (s, 1H, C_5–H pyrazole), 8.00–7.98 (d, 1H, C_5–H chromone, $J = 8.0$ Hz), 7.98–7.96 (d, 2H, Ph-CO, $J = 7.1$ Hz), 7.85–7.82 (dd, 2H, 3-phenylpyrazole, $J = 6.4$ and 2.8 Hz), 7.73–7.71 (d, 1H, C_8–H chromone, $J = 8.8$ Hz), 7.65–7.63 (d, 2H, 1-phenylpyrazole, $J = 7.2$ Hz), 7.58–7.54 (dd, 1H, C_6–H chromone, $J = 8.0$ and 7.2 Hz), 7.52–7.29 (m, 9H, Ar–H), 7.49–7.45 (dd, 1H, C_7–H chromone, $J = 8.4$ and 7.3 Hz), 7.39 (s, 1H, C_2–H chromone), 7.27 (s, 1H, CH=), 3.55 (s, 2H, CH_2). Anal. Calcd. for $C_{36}H_{26}N_4O_4$ (578.63): C, 74.73; H, 4.53; N, 9.68. Found: C, 74.42; H, 4.02; N, 9.61%.

3-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-1-(((4-oxo-4H-chromen-3-yl)methylene)amino)-5-phenyl-1,3-dihydro-2H-pyrrol-2-one (10) Yellow crystals, mp. 240–242 °C, yield 83%. IR (KBr, ν, cm^{-1}): 3056 (CH-aromatic), 1693 (C=O pyrrolone), 1653 (C=O chromone), 1620 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): (*anti* and *syn*-isomers, 3:1) 8.14–8.12 (d, 1H, C_5–H chromone, $J =$ 8.8 Hz), 8.08–8.03 (dd, C_7–H chromone, $J = 8.0$ and 7.0 Hz), 8.00–7.98 (d, C_8–H chromone, $J = 8.0$ Hz), 7.82–7.77 (dd, C_6–H chromone, $J = 8.8$ and 7.1 Hz), 7.72–7.30 (m, 15H, Ph-H); for *anti*-isomer: δ 9.56 (s, 1H, CH=N), 9.06 (s, 1H, C_5–H pyrazole), 8.67 (s, 1H, C_2–H chromone), 7.27 (s, 1H, CH=), 7.04 (s, 1H, C_4–H pyrrole); for *syn*-isomer: δ 9.28 (s, 1H, CH=N), 8.76 (s, 1H, C_5–H pyrazole), 8.62 (s, 1H, C_2–H chromone), 7.20 (s, 1H, CH=), 6.50 (s, 1H, C_4–H pyrrole). ^{13}C NMR (100 MHz, DMSO, δ, ppm): 90.67, 116.88, 119.16, 119.30, 119.50, 121.89, 123.84, 125.64, 125.75, 126.56, 126.77, 127.41, 128.21, 128.81, 128.99, 129.16, 129.34, 129.90, 132.49, 135.11, 139.53, 144.00, 144.12, 153.40, 155.02, 156.20 (C=N), 165.24 (C=O pyrrolone), 175.30 (C=O chromone). Anal. Calcd. for $C_{36}H_{24}N_4O_3$ (560.61): C, 77.13; H, 4.32; N, 9.99. Found: C, 76.89; H, 3.98; N, 9.91%.

Synthesis of 2-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-4-oxo-N′-(1-(2-oxo-2H-chromen-3-yl)ethylidene)-4-phenylbutanehydrazide (11)

A solution of the hydrazide **2** (2.11 g, 5 mmol) and 3-acetylcoumarin (0.94 g, 5 mmol) in dioxane (20 mL) was heated under reflux for 4 h. The reaction mixture was concentrated and then allowed to cool. The precipitated solid was collected by filtration and then recrystallized from ethanol/dioxane mixture (2:1) to furnish the coumarin derivative **11** as orange crystals, mp. 160–162 °C, yield 64%. IR (KBr, ν, cm^{-1}): 3058 (CH-aromatic), 2924 (CH-aliphatic), 1725 (C=O coumarin), 1696 (C=O ketone), 1651 (C=O amide), 1619 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.20 (s, 1H, C_5–H pyrazole), 8.43 (s, 1H, C_4–H coumarin), 8.08–7.82 (m, 4H, coumarin), 7.74–7.37 (m, 15H, Ph-H), 7.31 (s, 1H, CH=), 3.41 (s, 2H, CH_2), 2.07 (s, 3H, CH_3). EIMS (70 eV, *m/z*, %): 592.69 (M^+, 48.30), 569.38 (50.04), 535.73 (39.21), 454.20 (30.13), 340.40 (23.22), 242.40 (50.41), 193.70 (41.52), 156.84 (100.00), 121.16 (34.11), 106.80 (56.70), 77.16 (71.25). Anal. Calcd. for $C_{37}H_{28}N_4O_4$ (592.66): C, 74.99; H, 4.76; N, 9.45. Found: C, 74.62; H, 4.13; N, 9.38%.

Biological evaluation

Materials

Newcastle disease virus

NDV Genotype 7D NDV antigen accession No. KM288609 of 10^{10} EID_{50}/mL was obtained from Central Laboratory of veterinary Biologics (CLVB), Cairo, Egypt. Six compounds were checked (**3, 6, 7, 8, 10**, and **11**).

Embryonated specific pathogen free (SPF) chicken eggs (ECE_S)

One day old SPF. The eggs were obtained from the National Project for production of SPF eggs, Kom Oshim, Fayoum, Egypt. It was kept in the egg incubator at 37 °C with humidity 60–80% till the age of 9–11 days old.

Chicken erythrocytes Freshly collected chicken erythrocytes (1 and 10%) were prepared in saline solution after several washes in heamagglutination assay (HA).

SPF chicks Two hundred and twenty SPF chicks 1 day old were obtained from National Project for production of SPF eggs, Kom Oshim, Fayoum, Egypt. All birds were kept in biosafety isolators.

Newcastle disease virus vaccine Inactivated NDV vaccine its lab. Code: 1418198610 obtained from CLVB, Cairo, Egypt.

Methods

Antiviral activity

Screening of antiviral activity of different compounds was done using noncytotoxic concentration of it. Therefore, the investigation of different compounds as inhibitory agents against NDV replication (12-fold serial dilutions were performed) in SPF chicken embryos and their cytotoxicity was recorded.

Cytotoxicity (CC_{50})

Groups of 9–11 days SPF embryonated chicken eggs were inoculated with different concentration of each tested compound for calculation of cytotoxicity concentration at 50 (CC_{50}). Uninoculated SPF eggs were always included as control of embryo. The eggs inoculated via allantoic cavity and were incubated for 6 days PI at 37 °C with humidity 60–80%. CC_{50} of each test compound was determined as the concentration of compound that induced any embryos mortalities or any deviation than normal control embryos in 50% of embryonated chicken eggs.

Inhibition concentration (IC_{50})

Other group of SPF embryonated chicken eggs were inoculated with mixture of minimal cytotoxic concentration of different tested compounds with 10^{10} EID_{50}/mL of NDV (0.2 mL per egg) for calculation of the antiviral inhibitory concentration at 50 (IC_{50}). Uninoculated SPF eggs were always included as control of embryo. The eggs inoculated via allantoic cavity and were incubated for 6 days PI at 37 °C with humidity 60–80%. IC_{50} of tested compounds was assayed as the concentration of the compound that fully inhibited virus effect in 50% of embryonated chicken eggs.

Therapeutic index (TI)

The TI of the tested compound was expressed as CC_{50}/IC_{50} was calculated using the method described (Reed and Muench 1938).

In vitro antiviral assay

Nontoxic concentration of tested compounds (lower than CC_{50}) were checked for antiviral properties against NDV replication in SPF chicken embryos as follow:

(1) Mixing of 0.2 mL of the NDV and 0.2 mL of each compound separately and incubation for 1 h at ambient temperature.
(2) Twelvefold serial dilutions of the mixtures were performed.
(3) Groups (365 eggs) of 9–11-day-old SPF were inoculated by varying serial dilutions (from 1st dilution to 12th dilution, each dilution in five eggs) of each mixture (0.2 mL per egg) were inoculated via allantoic route.
(4) Daily examination of the inoculated eggs, deaths within 24 h post inoculation (PI) were discarded, and mortality between day 2 and 6, PI considered being specific.
(5) The NDV infectivity in ECE was determined by haemagglutinating activity of the allantoic fluid of the inoculating eggs as measured by the micro technique of the haemagglutinating (HA) test (Takasty 1966).
(6) Virus titer was calculated using the method (Reed and Muench 1938).
(7) Comparison of the known NDV titer with the antiviral activity.

Immune modulatory effect of different compounds in SPF chicks

Evaluation of immune boosting properties of different compounds (**3, 6, 7, 8, 10**, and **11**) was examined in one day old SPF chicks. A total of 221-day-old SPF chicks were divided into eight groups each contains thirty chicks (each group separated in biosafety isolator). At 7 days old, random samples from each group were examined for ND antibodies by HI test to check maternal antibodies. All chicks were proved to be free from ND antibodies by HI test.

First group (vaccinated only): consist of 30 experimental chicks were vaccinated with inactivated NDV vaccine. The vaccine was injected with 0.5 mL/bird subcutaneously in the dorsal region of the neck.

Second, third, fourth, fifth, sixth and seventh groups: each group consists of 30 chicks were vaccinated with inactivated NDV vaccine. Six milliliter from diluted nontoxic concentration of each tested compound (**3, 6, 7, 8, 10,** and **11**), respectively (each group separated in biosafety isolator) was added to the drinking water for six groups every day for 28 dpv.

Eighth group (10 chicks) was kept in separate isolator as nonvaccinated negative control.

Blood samples were collected individually from groups from jugular vein for estimation of the HI antibody titer in serum of vaccinated chicks at 28 dpv by Heamagglutination inhibition technique described (Allan and Gough 1974) using standard ND antigen (four HA units) with the comparison of humeral response of

vaccinated group and other vaccinated groups on the tested compound as immunostimulant. After collection of blood samples vaccinated groups and control group were challenged with a local isolated strain of NDV (Genotype 7D NDV antigen accession No. KM288609) containing at least 10^6 EID_{50}/bird (Code of American Federal Regulations National Archives and Records Administration 2012).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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