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Discovery of Dihydro-Alkyloxy-Benzyl-Oxopyrimidines as Promising Anti-Influenza Virus Agents

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A series of novel dihydro-alkyloxy-benzyl-oxopyrimidine derivatives were synthesized and evaluated for their activity against influenza virus in Madin–Darby canine kidney cells. Four dihydro-alkyloxy-benzyl-oxopyrimidine derivatives (4a1, 4a2, 4a3, and 4d1) showed potent activity against influenza virus. Among them, compound 4a3 was the most promising lead with broad activity against influenza A (antiviral EC_{50} values of 9 and 18 μM for the A/H1N1 and A/H3N2 subtype, respectively) and influenza B viruses (EC_{50}: 33 μM). The antiviral mechanism of action of these dihydro-alkyloxy-benzyl-oxopyrimidine derivatives must be quite different from that of the currently approved anti-influenza virus drugs that target the viral M2 or neuraminidase proteins. The dihydro-alkyloxy-benzyl-oxopyrimidine derivatives represent a new avenue for further optimization and development of novel anti-influenza virus agents.

Key words: anti-influenza agents, antiviral activity, DABO, influenza A, influenza B, influenza virus

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Human influenza viruses are highly contagious pathogens that affect the airway epithelia, resulting in acute respiratory symptoms with potentially severe or life-threatening complications in specific risk groups (1). It is estimated that, globally, influenza accounts for approximately 250 000–500 000 deaths during annual influenza epidemics[a]. Human influenza viruses also cause periodic pandemics such as seen in 1918, 1957, and 1968, and, more recently, 2009. In addition, highly pathogenic avian influenza viruses, such as the currently circulating avian A/H5N1 virus with a mortality of more than 60% in infected humans, continue to be a major threat to human health.

Influenza viruses are classified into three main types: A, B, and C. Further subdivision in subtypes (e.g. A/H1N1 and A/H3N2) is based on the structure of the hemagglutinin and neuraminidase (NA) antigens. Currently available drugs for the prophylaxis and treatment of influenza virus infections are the M2 ion channel blockers amantadine and rimantadine (Figure 1) and the NA inhibitors oseltamivir and zanamivir (Figure 1) (2,3). These four drugs are effective when administered early after virus infection or through prophylactic use. The usefulness of amantadine and rimantadine is limited because of their neurological side-effects, the lack of activity against influenza B virus, and the rapid emergence of drug-resistant virus mutants (3). The NA inhibitors oseltamivir and zanamivir have a more favorable safety profile and are active against all influenza (A and B) viruses. Unfortunately, global spread of oseltamivir-resistant A/H1N1 viruses has been described during the winter of 2008–2009 (4). Therefore, there is an urgent need for novel anti-influenza virus agents with a mechanism of action that differs from that of the existing drugs (5).

Recently, several dihydro-alkyloxy-benzyl-oxopyrimidine (DABO) (Figure 1) derivatives were reported as potent non-nucleoside reverse transcriptase inhibitors (NNRTIs) of HIV-1, exerting robust antiviral activity against both the wild-type HIV-1 and a panel of clinically relevant HIV-1 mutants bearing mutations in the RT enzyme (6). Few studies have been carried out to evaluate the potential activity of DABO compounds against other viruses than HIV-1, such as influenza virus. We here report on the chemical synthesis, anti-influenza A and B virus activity, and structure–activity relationship of novel DABO derivatives. The potent anti-influenza virus activity of selected DABO compounds creates an opportunity to design novel anti-influenza virus agents that could be helpful to combat influenza virus mutants with resistance to the existing M2 or NA inhibitors.

Materials and Methods

Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Nexus 470FT-IR Spectrometer. NMR spectra were obtained on a Brucker Avance-600 NMR-spectrometer in the indicated solvents. Chemical shifts are expressed in δ units and TMS as internal

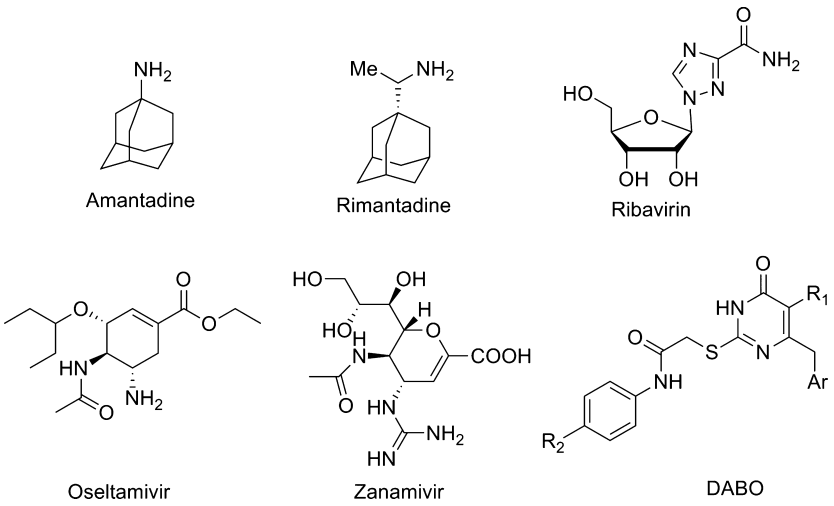


Figure 1: Structures of the existing anti-influenza drugs and the novel dihydro-alkyloxy-benzyl-oxopyrimidine derivatives.

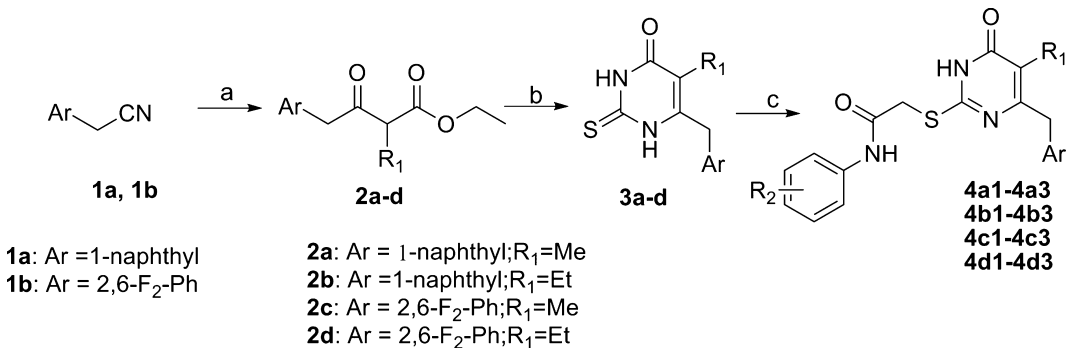
reference. Mass spectra were taken on a LC Autosampler Device: Standard G1313A instrument. Thin layer chromatography (TLC) was performed on silica gel GF254 for TLC (Merck, Darmstadt, Germany), and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure. Compounds were prepared using the routes shown in Scheme 1.

General procedure for the preparation of *β*-ketoesters 2a–d

The activated zinc dust was prepared by washing zinc dust sequentially with 3 N aq. HCl, distilled water, ethanol, and ether and drying under vacuum. Activated zinc dust (32.5 g, 500 mmol) was suspended in dry tetrahydrofuran (THF) (200 mL) and refluxed under a nitrogen atmosphere. A few drops of 2-bromoalkanoates were added to initiate the reaction. After the appearance of a green color, arylacetonitriles (100 mmol) were added in one portion followed by the dropwise addition of 2-bromoalkanoates (260 mmol) over 1 h. The reaction mixture was refluxed for an additional 2 h, diluted with THF (600 mL), and quenched with aq K_2CO_3 (50%, 125 mL). Rapid stirring for 60 min gave two distinct layers. The THF layer was decanted, the residue was washed with THF (150 mL), and the combined THF fractions were treated with aq. HCl (10%, 150 mL) at room temperature for 45 min. The mixture was concentrated under reduced pressure, diluted with CH_2Cl_2 (300 mL), and washed with saturated $NaHCO_3$ (3 × 350 mL) and brine (3 × 350 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated to give the crude products **2a**–**d**, respectively, which were directly used in the following step without further purification.

General procedure for the preparation of 5-alkyl-6-substituted thiouracil 3a–d

Sodium metal (8.2 g, 356 mmol) was dissolved in absolute ethanol (50 mL), and thiourea (19 g, 249 mmol) and *β*-ketoesters **2a**–**d** (178 mmol) were added to the clear solution at room temperature. The reaction mixture was refluxed for 6–12 h (checked by TLC) under a nitrogen atmosphere. The reaction mixture was cooled to room temperature. Then, solvent was evaporated, and the residues were dissolved in H_2O and were precipitated by addition of conc. aq. HCl and subsequent acidification to pH 4 with glacial AcOH. The resulting precipitate was filtered under reduced pressure. The solid was washed sequentially with H_2O, EtOH, and Et_2O and then dried to give **3a**–**d**[b] (7), which is directly used in the next step without further purification.



Scheme 1: Reagents and conditions: (a) (1) R1CHBrCOOEt, Zn, THF, reflux, (2) 50% K_2CO_3, (3) 13% HCl; (b) thiourea, EtONa, reflux, 6–12 h; (c) appropriate N-phenylacetamide halides, K_2CO_3, DMF, rt, 12 h.

General procedure for the preparation of target compounds 4a–d

cCompounds **3a**–**d** (5 mmol) and appropriate *N*-phenylacetamide halides (5.5 mmol) were suspended in anhydrous DMF (25 mL) in the presence of anhydrous potassium carbonate (0.759 g, 5.5 mmol) at room temperature. The mixtures were irradiated at room temperature for 12 h. The reaction mixture was poured into cold H_2O (200 mL); the resulting precipitate was collected by filtration under reduced pressure and washed sequentially with H_2O, EtOH, and Et_2O and then dried to give the corresponding crude product, which was purified by crystallization to give the pure target compounds **4a**–**d**.

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-methylpyrimidin-4(3H)-one (4a1)

Recrystallized from EtOH-DMF as a white crystal, Yield: 23.6%, mp: 236–238 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.74 (s, 1H, NH), 10.18 (s, 1H, NH), 8.10–7.10 (m, 11H), 4.28 (s, 2H, *S*-CH_2), 3.92 (s, 2H, CH_2), 1.87 (s, CH_3, 3H). IR (KBr, ⁄cm): 3267 (υ_{NH}), 3117 (υ_{NH}), 1663 ($\upsilon_{C=O}$), 1268 ($\upsilon_{C–N}$), 1244 ($\upsilon_{C–N}$). ESI-MS: *m/z* 450.5 [M+H], 472.4 [M+Na]. $C_{24}H_{20}ClN_3O_2S$ (449.1).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-methylpyrimidin-4(3H)-one (4a2)

Recrystallized from EtOH-DMF as a white crystal, Yield: 23.9%, mp: 240–242 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.82 (s, 1H, NH), 10.19 (s, 1H, NH), 8.11–7.10 (m, 11H), 4.26 (s, 2H, *S*-CH_2), 3.91 (s, 2H, CH_2), 1.87 (s, CH_3, 3H). IR (KBr, ⁄cm): 3259 (υ_{NH}), 3045 (υ_{NH}), 1651 ($\upsilon_{C=O}$), 1267 ($\upsilon_{C–N}$), 1244 ($\upsilon_{C–N}$). ESI-MS: *m/z* 494.1 [M+H], 516.1 [M+Na]. $C_{24}H_{20}BrN_3O_2S$ (493.05).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-methylpyrimidin-4(3H)-one (4a3)

Recrystallized from EtOH-DMF as a white crystal, Yield: 24.2%, mp: 239–241 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.78 (s, 1H, NH), 9.93 (s, 1H, NH), 8.16 (d, *J* = 8.4Hz, 1H), 7.91 (d, *J* = 7.8Hz, 1H), 7.75 (d, *J* = 8.4Hz, 1H), 7.54–7.15 (m, 6H), 6.80 (d, *J* = 8.4Hz, 2H), 4.30 (s, 2H, *S*-CH_2), 3.90 (s, 2H, CH_2), 3.70 (s, OCH_3, 3H), 1.89 (s, CH_3, 3H).IR (KBr, ⁄cm): 3290 (υ_{NH}), 3042 (υ_{NH}), 1653 ($\upsilon_{C=O}$), 1299 ($\upsilon_{C–N}$), 1246 ($\upsilon_{C–N}$). ESI-MS: *m/z* 446.2 [M+H], 468.2 [M+Na]. $C_{25}H_{23}N_3O_3S$ (445.15).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-ethylpyrimidin-4(3H)-one (4b1)

Recrystallized from EtOH-DMF as a white crystal, Yield: 25.7%, mp: mp: 217–219 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.78 (s, 1H, NH), 10.17 (s, 1H, NH), 8.12 (d, *J* = 7.8Hz, 1H), 7.88 (d, *J* = 7.2Hz, 1H), 7.71(d, *J* = 7.8Hz, 1H), 7.53–7.09 (m, 8H), 4.29 (s, 2H, *S*-CH_2), 3.92 (s, 2H, CH_2), 2.37 (q, *J* = 7.2 Hz, CH_2CH_3, 2H), 0.84 (t, *J* = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, ⁄cm): 3271 (υ_{NH}), 3045 (υ_{NH}), 1659 ($\upsilon_{C=O}$), 1271 ($\upsilon_{C–N}$), 1259 ($\upsilon_{C–N}$). ESI-MS: *m/z* 464.2 [M+H], 486.2 [M+Na]. $C_{25}H_{22}ClN_3O_2S$ (463.11).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-ethylpyrimidin-4(3H)-one (4b2)

Recrystallized from EtOH-DMF as a white crystal, Yield: 28.7%, mp: 224–226 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.93 (s, 1H, NH), 10.17 (s, 1H, NH), 8.11–7.08 (m, 11H), 4.28 (s, 2H, *S*-CH_2), 3.91 (s, 2H, CH_2), 2.38 (q, *J* = 7.2 Hz, CH_2CH_3, 2H), 0.83 (t, *J* = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, ⁄cm): 3253 (υ_{NH}), 3043 (υ_{NH}), 1652 ($\upsilon_{C=O}$), 1269 ($\upsilon_{C–N}$), 1244 ($\upsilon_{C–N}$). ESI-MS: *m/z* 408.1 [M+H], 530.1 [M+Na]. $C_{25}H_{22}BrN_3O_2S$ (507.06).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-ethylpyrimidin-4(3H)-one (4b3)

Recrystallized from EtOH-DMF as a white crystal, Yield: 22.9%, mp: 237–239 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.95 (s, 1H, NH), 10.09 (s, 1H, NH), 8.14–7.06 (m, 11H), 4.21 (s, 2H, *S*-CH_2), 3.94 (s, 2H, CH_2), 3.76 (s, OCH_3, 3H), 2.39 (q, *J* = 7.2 Hz, CH_2CH_3, 2H), 0.85 (t, *J* = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, ⁄cm): 3255 (υ_{NH}), 3130 (υ_{NH}), 1650 ($\upsilon_{C=O}$), 1291 ($\upsilon_{C–N}$), 1251 ($\upsilon_{C–N}$).ESI-MS: *m/z* 460.2 [M+H], 482.2 [M+Na]. $C_{26}H_{25}N_3O_3S$ (459.16).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3H)-one (4c1)

Recrystallized from EtOH-DMF as a white crystal, Yield: 27.1%, mp: 248–250 °C (dec). ^{1}H NMR (DMSO-d6, ppm) δ: 12.86 (s, 1H, NH), 10.15 (s, 1H, NH), 7.54 (d, *J* = 9Hz, 2H), 7.37 (d, *J* = 9Hz, 2H), 7.15–6.85 (m, 3H), 3.85 (s, 2H, *S*-CH_2), 3.80 (s, 2H, CH_2), 2.00 (s, CH_3, 3H). IR (KBr, ⁄cm): 3262 (υ_{NH}), 3044 (υ_{NH}), 1658 ($\upsilon_{C=O}$), 1267 ($\upsilon_{C–N}$), 1247 ($\upsilon_{C–N}$). ESI-MS: *m/z* 436.4 [M+H]. $C_{20}H_{16}ClF_2N_3O_2S$ (435.06).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3H)-one (4c2)

Recrystallized from EtOH-DMF as a white crystal, Yield: 26.6%, mp: 251–253 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.72 (s, 1H, NH), 10.10 (s, 1H, NH), 7.50–6.87 (m, 7H), 3.85 (s, 2H, *S*-CH_2), 3.82 (s, 2H, CH_2), 2.00 (s, CH_3, 3H). IR (KBr, ⁄cm): 3261 (υ_{NH}), 3041(υ_{NH}), 16594($\upsilon_{C=O}$), 1267 ($\upsilon_{C–N}$), 1247 ($\upsilon_{C–N}$). ESI-MS: *m/z* 480.2 [M+H], 502.2 [M+Na]. $C_{20}H_{16}BrF_2N_3O_2S$ (479.01).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3H)-one (4c3)

Recrystallized from EtOH-DMF as a white crystal, Yield: 23.5%, mp: 233–235 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ:12.74 (s, 1H, NH), 9.83 (s, 1H, NH), 7.42–6.87 (m, 7H), 3.87 (s, 2H, *S*-CH_2), 3.77 (s, 2H, CH_2), 3.60 (s, OCH_3, 3H), 2.00 (s, CH_3, 3H). IR (KBr, ⁄cm): 3257

(υ_{NH}), 3050 (υ_{NH}), 1656 ($\upsilon_{C=O}$), 1248 (υ_{C-N}). ESI-MS: m/z 432.4 [M+H], 454.3 [M+Na]. $C_{21}H_{19}F_2N_3O_3S$ (431.11).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3H)-one (4d1)

Recrystallized from EtOH-DMF as a white crystal, Yield: 26.6%, mp: 222–224 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.73 (s, 1H, NH), 10.10 (s, 1H, NH), 7.54 (d, J = 9Hz, 2H), 7.37 (d, J = 9Hz, 2H), 7.10–6.85 (m, 3H), 3.86 (s, 2H, S-CH_2), 3.80 (s, 2H, CH_2), 2.51 (q, J = 7.2 Hz, CH_2CH_3, 2H), 1.01(t, J = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, /cm): 3288 (υ_{NH}), 3054 (υ_{NH}), 1673 ($\upsilon_{C=O}$), 1650 ($\upsilon_{C=O}$), 1269 (υ_{C-N}), 1244 (υ_{C-N}). ESI-MS: *m/z* 450.3 [M+H]. $C_{21}H_{18}ClF_2N_3O_2S$ (449.08).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3H)-one (4d2)

Recrystallized from EtOH-DMF as a white crystal, Yield: 23.1%, mp: 223–225 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.70 (s, 1H, NH), 10.09 (s, 1H, NH), 7.50 (d, J = 9Hz, 2H), 7.47 (d, J = 9Hz, 2H), 7.10–6.85 (m, 3H), 3.86 (s, 2H, S-CH_2), 3.80 (s, 2H, CH_2), 2.53 (q, J = 7.2 Hz, CH_2CH_3, 2H), 1.01(t, J = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, /cm): 3284 (υ_{NH}), 3056 (υ_{NH}), 1657 ($\upsilon_{C=O}$), 1266 (υ_{C-N}), 1243 (υ_{C-N}). ESI-MS: *m/z* 494.2 [M+H]. $C_{21}H_{18}BrF_2N_3O_2S$ (493.03).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3H)-one (4d3)

Recrystallized from EtOH-DMF as a white crystal, Yield: 27.1%, mp: 232–234 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.66(s, 1H, NH), 9.85 (s, 1H, NH), 7.42–6.87 (m, 7H), 3.89 (s, 2H, S-CH_2), 3.76 (s, 2H, CH_2), 3.72(s, OCH_3, 3H), 2.51 (q, J = 7.2 Hz, CH_2CH_3, 2H), 1.02(t, J = 7.2 Hz, CH_2CH_3, 3H). IR (KBr, /cm): 3250 (υ_{NH}), 3050 (υ_{NH}), 1656 ($\upsilon_{C=O}$), 1241 (υ_{C-N}). ESI-MS: *m/z* 446.3 [M+H], 468.3 [M+Na]. $C_{22}H_{21}F_2N_3O_3S$ (445.13).

Antiviral activities in influenza A/H1N1, A/H3N2, and B virus-infected Madin–Darby canine kidney (MDCK) cells

Madin–Darby canine kidney cells (8) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 1 mM sodium pyruvate, and 0.075% sodium bicarbonate. During virus experiments, the MDCK cells were maintained in Ultra MDCK medium (Lonza, Basel, Switzerland), supplemented with 0.0225% sodium bicarbonate, 2 mM L-glutamine, and 2 μg/mL tosylphenylalanylchloromethylketon-treated trypsin (Sigma, St Louis, MO, USA). The human influenza virus strains A/PR/8/34 (A/H1N1) and B/HK/5/72 were purchased from the American Type Culture Collection (ATCC). The strain A/HK/7/87 (A/H3N2) was obtained from J. Neyts (Katholieke Universiteit Leuven, Leuven, Belgium). Virus stocks of these laboratory-adapted strains were prepared by passaging them in 10-day-old embryonated chicken eggs.

The following control compounds were included: ribavirin (Virazole®), obtained from ICN Pharmaceuticals; amantadine (Sigma); rimantadine (Sigma); and oseltamivir carboxylate (GS-4071) (a kind gift from T. Cihlar, Gilead Sciences, Foster City, CA, USA). The test compounds were evaluated for anti-influenza virus activity by a multicycle cytopathic effect (CPE) reduction assay. MDCK cells were seeded into 96-well plates at 7500 cells per well 16 h prior to infection and incubated at 35 °C. Serial dilutions of the compounds were added to the cells, together with the influenza virus (multiplicity of infection, fifty 50% cell culture infective doses [$CCID_{50}$] per well, which corresponds to 0.0004 PFU per cell). At 3 days postinfection (p.i.), microscopy was performed to determine the antiviral activity, expressed as the compound concentration producing 50% inhibition of virus-induced CPE [50% effective concentration (EC_{50})], as well as the cytotoxicity of the compounds, expressed as the compound concentration causing minimal changes in cell morphology (MCC). The data were confirmed by the formazan-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega, Madison, WI, USA), and the spectrophotometric data were used to calculate the EC_{50} and 50% cytotoxic concentration (CC_{50}).

Neuraminidase inhibition assay

Four compounds were evaluated for *in vitro* inhibitory actions using the method reported by Guanhua Du and, as the source of NA, the influenza virus strain A/H1N1 [strain A/PR/8/34] described by Laver. This spectrofluorometric assay (excitation wavelength: 355 nm and emission wavelength: 460 nm) uses 2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid (MUNANA) as the enzyme substrate, which is converted to a fluorescent product upon cleavage by NA. The intensity of fluorescence can reflect the activity of NA sensitively.

In the enzyme reaction system, there were 30 μL of the enzyme in 33 mM MES buffer (pH 3.5), 10 μL of 4 mM $CaCl_2$, 20 μL of 20 μM MUNANA, and 30 μL water in a 96-well microplate. The terminal volume was 100 μL. After 10 min at 37 °C, 150 μL of 14 mM NaOH in 83% ethanol was added to 0.1 mL of the reaction mixture to terminate the reaction. The intensity of the fluorescence was quantitated in Fluostar Galaxy (excitation, 360 nm; emission, 450 nm), and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition versus the inhibitor concentration, and determination of each point was performed in duplicate.

Results and Discussion

Chemistry

Compounds were prepared using the routes shown in Scheme 1. The β-ketoesters (**2a**–**d**) were prepared by a simple and high-yielding method of Hannick and Kishi, through the reaction of arylacetonitriles (**1a**–**b**) with activated zinc dust and 2-bromoalkanoates (9,10). Subsequent condensation of β-ketoesters (**2a**–**d**) with thiourea in the presence of EtONa in refluxing ethanol gave the key intermediates 5-alkyl-6-substituted thiouracil **3a**–**d** (11). Next,

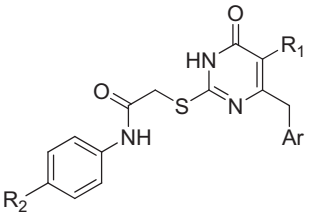
selective *S*-alkylation of **3a**–**d** with the appropriate *N*-phenylacetamide halides (1:1.1) in the presence of K_2CO_3 in anhydrous DMF afforded the DABO compounds **4a**–**d**. The analytical and spectral data of the all compounds were in full agreement with the proposed structures.

Antiviral activities in influenza A/H1N1, A/H3N2, and B virus-infected MDCK cells

The compounds were evaluated for antiviral activity against human influenza viruses of the A/H1N1 (strain A/PR/8/34), A/H3N2 (strain A/HK/7/87), and B (strain B/HK/5/72) (sub)type, using a cell-culture-based assay in MDCK cells, as previously described (12,13). Antiviral activity was expressed as the 50% antivirally effective concentration (EC_{50}) or compound concentration producing 50% inhibition of virus-induced CPE, as determined by microscopy or MTS assay. Cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}), or compound concentration causing 50% cytotoxicity in the MTS assay, and the MCC (minimal cytotoxic concentration), representing the compound concentration at which MCC were observed (12,13). In all experiments, oseltamivir carboxylate, ribavirin, amantadine, and rimantadine were used as the reference compounds. The data obtained are shown in Table 1.

Four DABO compounds exhibited good activity against influenza virus. The most active and selective DABO derivative was compound **4a3**, which had broad anti-influenza virus activity, with antiviral EC_{50} values (i.e. average values from both the CPE and MTS assay) of 9, 18, and 33 μM, for influenza A/H1N1, A/H3N2, and influenza B virus, respectively. Moreover, compound **4a3** displayed no cytotoxicity at the highest concentration tested (224 μM), thus yielding a selectivity index (defined as the ratio of MCC to EC_{50}) of at least 7 (for influenza B virus) or at least 12–25 (for influenza A/H1N1 and A/H3N2). In this respect, **4a3** was superior to the reference compound ribavirin, which had a lower selectivity index (~11) than **4a3**. Interestingly, **4a3** showed favorable activity against the A/PR/8/34 (A/H1N1) strain, which carries two amantadine resistance mutations in the M2 protein, as reflected in its high EC_{50} val-

Table 1: Anti-influenza virus activity of dihydro-alkyloxy-benzyl-oxopyrimidine derivatives in Madin–Darby canine kidney cells



				Antiviral EC_{50}[a] (μM)						Cytotoxicity (μM)	
				Influenza A/H1N1		Influenza A/H3N2		Influenza B			
Cpd No.	Ar	R_1	R_2	CPE	MTS	CPE	MTS	CPE	MTS	CC_{50}[b]	MCC[c]
4a1	1-naphthyl	CH_3	Cl	>222	>222	21	22	>222	>222	>222	133
4a2	1-naphthyl	CH_3	Br	>202	>202	55	44	>202	>202	>202	≥20
4a3	1-naphthyl	CH_3	OCH_3	9.0	9.0	26	9.7	≥45	≥22	>224	>224
4b1	1-naphthyl	CH_2CH_3	Cl	>216	>216	>216	>216	>216	>216	11	≥8.6
4b2	1-naphthyl	CH_2CH_3	Br	>197	>197	>197	>197	>197	>197	18	7.9
4b3	1-naphthyl	CH_2CH_3	OCH_3	>218	>218	>218	>218	>218	>218	>218	≥8.7
4c1	2,6-F_2-Ph	CH_3	Cl	>229	>229	>229	>229	>229	>229	>229	>229
4c2	2,6-F_2-Ph	CH_3	Br	>209	>209	>209	>209	>209	>209	>209	≥42
4c3	2,6-F_2-Ph	CH_3	OCH_3	>232	>232	>232	>232	>232	>232	>232	≥46
4d1	2,6-F_2-Ph	CH_2CH_3	Cl	133	≥22	27	16	>222	>222	>222	≥44
4d2	2,6-F_2-Ph	CH_2CH_3	Br	>202	>202	>202	>202	>202	>202	>202	≥40
4d3	2,6-F_2-Ph	CH_2CH_3	OCH_3	>224	>224	>224	>224	>224	>224	>224	45
Oseltamivir carboxylate				2.6	4.7	1.7	2.4	>100	>100	>100	>100
Ribavirin				10	9.7	10	9	9	8.4	>100	≥100
Amantadine				134	149	2.4	3.9	>500	>500	>500	>500
Rimantadine				32	34	0.15	0.05	>500	>500	258	500

MCC, minimal changes in cell morphology.

[a]Antiviral activity was expressed as the EC_{50} value, defined as the compound concentration producing 50% inhibition of virus replication, as estimated by microscopic scoring of the cytopathic effect (CPE), or by measuring cell viability in the formazan-based MTS assay. Values are the mean of duplicate experiments.

[b]50% cytotoxic concentration, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.

[c]Minimum compound concentration that causes a microscopically detectable alteration of normal cell morphology.

ues for the M2 inhibitors amantadine and rimantadine (Table 1). Finally, **4a3** was also found to have more consistent anti-influenza virus activity than oseltamivir carboxylate, an NA inhibitor for which the outcome in cell-culture-based antiviral assays is prone to some variation (14).

Two related compounds, **4a1** and **4a2**, had moderate activity against influenza A/H3N2 (EC_{50}: 22 and 50 μM, respectively). Compound **4d1** also showed activity against both influenza A/H1N1 and A/H3N2 [average EC_{50} values: 78 μM (A/H1N1) and 22 μM (A/H3N2)]. For these four active compounds, the 50% cytotoxic concentrations determined by MTS assay were higher than ~200 μM (the highest concentration tested). Microscopical evaluation demonstrated that **4a1**, **4a2**, **4a3**, and **4d1** caused MCC at compound concentrations of 133, 20, >224, and 44 μM, respectively.

Structure-activity relationship (SAR) analysis on this series of compounds demonstrates that the anti-influenza virus activity of these DABO derivatives is influenced by the substituent at the *C*-6 position of the pyrimidine ring. Compounds with the large side chain (1-naphthylmethyl at *C*-6) showed better inhibitory activity than those with small ones (2,6-difluorobenzyl analogs), and these results indicate that steric factors influence the inhibitory activity. The nature of the R_2 substituent at the phenyl ring of the *C*-2 side chain also influenced the anti-influenza virus activity of these DABO derivatives, the order being (from high to low activity) 4-OCH_3 > 4-Cl > 4-Br, thus emphasizing the importance of having an electron-donating group at the phenyl moiety. Replacement of the R_1 methyl at the *C*-5 position of the pyrimidine ring by an ethyl group leads to compounds devoid of anti-influenza virus activity. The only exception to this limited SAR analysis was **4d1**, which showed activity against influenza A/H3N2. From the above, the antiviral activity of DABO derivatives appears to depend on different factors: electronic properties [compound **4a3** with an electron-donating group on the *C*-2 side chain phenyl is clearly more active than the corresponding derivatives **4a1** and **4a2** with an electron-withdrawing substituent] and steric bulkiness [6-(1-naphthylmethyl) analogs are more active than the 6-(2,6-difluorobenzyl) analogs].

Neuraminidase inhibition assay

With regard to the antiviral mechanism of action, we are currently trying to identify the target of the four active DABO compounds. They are effective against both influenza A and B type viruses, implying that their mechanism of action differs from that of the M2 ion channel blockers amantadine and rimantadine, which are known to be inactive against influenza B virus.

In addition, we verified whether these DABO derivatives may be acting on the viral NA. The enzymatic NA inhibitory assay was performed using the method reported by Guanhua Du (15) and, as the source of NA, the influenza virus strain A/H1N1 [strain A/PR/8/34] described by Laver (16). The compound concentration producing 50% inhibition of NA activity (IC_{50}) was calculated by plotting the percentage inhibition versus the inhibitor concentration, with each data point performed in duplicate. The results showed that the DABO compounds had no inhibitory effect on NA (Table 2).

Table 2: Evaluation of the dihydro-alkyloxy-benzyl-oxopyrimidine derivatives for inhibitory effect on influenza virus neuraminidase[a]

Cpd. No.	Concentration	Inhibition ratio (%)	IC_{50}[b]
4a1	89 μM	16	–
4a2	81 μM	11	–
4a3	90 μM	7	–
4d1	89 μM	6	–
Zanamivir	12 nM	99	0.29 nM

[a]Influenza virus strain used: strain A/PR/8/34.
[b]"–" means that the IC_{50} could not be calculated.

Conclusions

In conclusion, a series of novel DABO derivatives were synthesized and found to have broad anti-influenza virus activity. The action mechanism of DABO derivatives appears to be quite different from that of currently approved anti-influenza virus drugs. These DABO inhibitors represent a new avenue for future research and development of novel anti-influenza drugs. Further studies on molecular modification and specific target identification of these DABO derivatives are underway in our laboratories.

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Notes

[a]World Health Organization reported on 24 February 2010. Available from: http://www.who.int/.

[b]**3a**, Mp 218–220 °C; **3b**, Mp 201–203 °C; **3c**, Mp 279–281 °C (dec); **3d**, Mp 248–250 °C (dec).