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(−)-Carbodine: Enantiomeric synthesis and in vitro antiviral activity against various strains of influenza virus including H5N1 (avian influenza) and novel 2009 H1N1 (swine flu)

Jagadeeshwar R. Rao [a], Ashok K. Jha [a], Ravindra K. Rawal [a], Ashoke Sharon [a], Craig W. Day [b], Dale L. Barnard [b], Donald F. Smee [b], Chung K. Chu [a,*]

[a] The University of Georgia College of Pharmacy, Athens, GA 30602, USA
[b] Utah State University Institute for Antiviral Research, Logan, UT, USA

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ABSTRACT

Enantiomerically pure cyclopentyl cytosine [(−)-carbodine **1**] was synthesized from D-ribose and evaluated for its anti-influenza activity in vitro in comparison to the (+)-carbodine, (±)-carbodine and ribavirin. (−)-Carbodine **1** exhibited potent antiviral activity against various strains of influenza A and B viruses.

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In recent years the emergence of the avian influenza A virus subtype H5N1 has raised concerns for public health worldwide. Since January 2004, the World Health Organization (WHO) has been tracking the human cases of avian influenza A (H5N1), and up to December 30, 2009, 282 deaths (467 cases; 60.4% mortality) have been reported.[1] Recently a new pandemic influenza A (Novel H1N1 2009) virus originally called 'swine flu' has also emerged which has never been circulated before among humans. WHO officially raised the phase of pandemic alert to level 6 with regard to the novel H1N1 2009 influenza on June 11, 2009. As of January 10, 2010, more than 208 countries have reported the laboratory confirmed cases of the novel influenza H1N1 2009 strains, including at least 13,554 deaths.[2] The pandemic is unfolding in the southern hemisphere and WHO is nervously monitoring the coming winter wave in the northern hemisphere.

The use of neuraminidase inhibitors (oseltamivir and zanamavir) for the therapy as well as the prophylaxis of influenza A and B virus infections has been considered a new 'millennium conundrum' and the stockpiling of oseltamivir has been implemented worldwide in preparation of an influenza pandemic due to the recent emergence of H5N1 virus. However, the emergence of drug resistant of H5N1 virus strains with reduced susceptibility to oseltamivir[3] as well as sporadic cases of oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection may potentially limit its clinical use in the future.[2b] Furthermore, oseltamivir is the only orally active anti-influenza agent. Therefore, there is an urgent need for additional orally effective novel antiviral agents and potentially effective against drug resistant influenza viruses, as well as agents with different mode of action.

Natural as well as synthetic carbocyclic nucleosides are well known for their interesting biological properties, including antitumor as well as antiviral activity against a wide variety of RNA and DNA viruses.[4] The carbocyclic analogue of cytosine (carbodine **1**;[5] Fig. 1) was previously synthesized as a racemic mixture and has been shown to possess significant antitumor (lymphoid leukemia L1210 in mice) and antiviral activities against human influenza type A virus, measles, respiratory syncytial virus, rotavirus, Venezuelan Equine Encephalitis (VEE) virus, Herpes Simplex Viruses (HSV-1 and HSV-2).[6] Carbodine is metabolized to its triphosphate

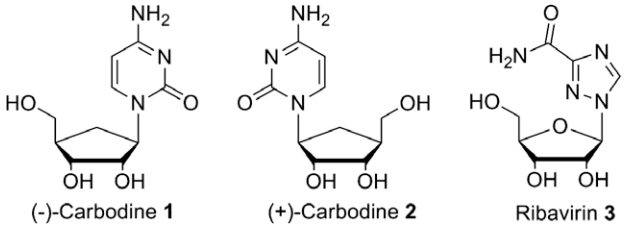

Figure 1. The chemical structure of carbodine and ribavirin.

* Corresponding author. Tel.: +1 (706) 542 5379; fax: +1 (706) 542 5381.
E-mail address: DCHU@mail.rx.uga.edu (C.K. Chu).

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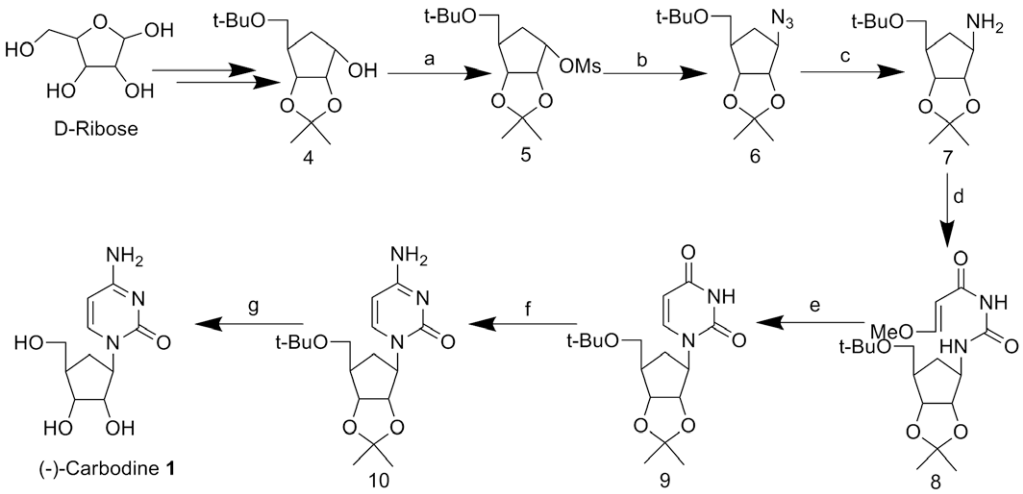

Scheme 1. Reagents and conditions: (a) $MeSO_2Cl$, TEA, DCM, rt, quantitative; (b) NaN_3, DMF, 140 °C, 4 h, 89%; (c) 10% Pd/C, EtOH, rt, 30 psi, 2 h, quantitative; (d) β-methoxyacryloyl isocyanate, DMF, −20 °C to rt, 10 h, 72%; (e) 30% NH_4OH, EtOH/dioxane (1:1), 100 °C, 18 h, 85%; (f) 2,4,6-triisopropylbenzenesulfonyl chloride, DMAP, Et_3N, 30% NH_4OH, rt, 17 h, 78%; (g) TFA/H_2O (2:1), 60 °C, 3 h, 82%.

in mammalian cells and interferes with the viral RNA-dependent RNA polymerase reaction, which may be the principal mode of action for its biological activiy.[6c,7] These interesting biological properties of carbodine prompted us to synthesize enantiomerically pure (−)-carbodine (**1**, Fig. 1) for antiviral evaluations in comparison to its racemic and (+)-enantiomer **2**.

Ohno[5d] synthesized (−)-carbodine using enzymatic hydrolysis as the key step for the synthesis of the cyclopentyl intermediate, whereas Russ et al.[5e] explored by the double bond reduction of cyclopentenyl cytosine, which gave a varying ratio of (−)-carbodine and isocarbodine. Both of these methods resulted in poor yield and selectivity. Herein, we report the practical synthesis and antiviral activity of carbodine against various strains of influenza viruses of the current interest.

The D-(−)-carbodine **1** was synthesized by the linear method from the cylopentanol key intermediate **4** (Scheme 1), which was obtained from D-ribose in good yield using previously reported method by our group for L-(+)-carbodine.[8] We initially attempted a convergent approach to couple N^6-protected cytosine to compound **4** under the Mitsunobu reaction condition which led to mixture of *N*1 (minor)- and *O*2 (major)-regioisomers.[9] Therefore, we used a linear approach[8] to synthesize functionalized cyclopentylamine **7**, then constructed the heterocyclic base in a step-wise manner. Compound **4** was reacted with mesyl chloride followed by S_N2 reaction with sodium azide. Reduction of the azide **6** using Pd/C hydrogenation gave cyclopentyl amine **7**[10] in quantitative yield, which was reacted with β-methoxyacryloyl isocyanate followed by heating with NH_4OH to obtain the target uracil com-

Table 1
Anti-influenza in vitro activity of (−)-carbodine, (+)-carbodine, (±)-carbodine and ribavirin against various H5N1 strains

Virus type	Inoculum[b]	Compound	Anti-influenza activity (H5N1)[a]						
			EC_{50}[c] (μM)		EC_{90}[d] (μM)	IC_{50}[e] (μM)	SI[f]		
			Visual CPE	Neutral Red	Virus Yield Reduction		Visual CPE	Neutral Red	Virus Yield Reduction
Duck H5N1	60 ± 46	(−)-Carbodine	1.8 ± 0.5	2.0 ± 1.4	10.5 ± 10.1	>414	>230	>207	>39
		(+)-Carbodine	>414	>414	>414	>414	1	1	1
		(±)-Carbodine	2.4 ± 0.9	5.0 ± 1.9	17.6 ± 13.8	>414	>173	>83	>24
		Ribavirin	35.4 ± 11.0	32.5 ± 8.6	43.6 ± 15.0	>1310	>37	>40	>30
Gull H5N1	53 ± 40	(−)-Carbodine	0.8 ± 0.3	1.0 ± 0.3	1.6 ± 1.0	>414	>518	>414	>259
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	1.1 ± 0.6	1.4 ± 0.5	1.6 ± 0.5	>414	>376	>296	>259
		Ribavirin	23.6 ± 1.7	27.4 ± 6.8	20.3 ± 4.7	>1310	>55	>48	>65
Hong Kong H5N1	84 ± 91	(−)-Carbodine	0.7 ± 0.1	0.8 ± 0.04	2.1 ± 0.4	>414	>591	>518	>197
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	0.7 ± 0.2	1.8 ± 1.0	2.0 ± 0.06	>414	>591	>230	>207
		Ribavirin	4.3 ± 0.9	9.4 ± 0.6	16.8 ± 14.3	>1310	>305	>139	>78
Vietnam H5N1	139 ± 156	(−)-Carbodine	0.8 ± 0.2	1.5 ± 0.9	5.7 ± 5.4	>414	>518	>276	>73
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	1.0 ± 0.4	2.9 ± 2.3	12.1 ± 9.3	>414	>414	>143	>34
		Ribavirin	8.4 ± 5.9	13.9 ± 10.0	11.6 ± 9.8	>1310	>156	>94	>113

[a] Average of triplicate experimental data (duplicate for Hong Kong).
[b] Approximate $CCID_{50}$ inoculated per well, average ± SD from triplicate assays.
[c] Effective concentration required to reduce influenza virus-induced cytopathic effect by 50%.
[d] Concentration required to reduce infectious virus titer by 90%.
[e] Concentration of drug for 50% cell inhibition without virus.
[f] In vitro selectivity index (IC_{50}/EC_{50}), and for virus yield reduction (IC_{50}/EC_{90}).

Table 2
Anti-influenza *in vitro* activity of (−)-carbodine, (+)-carbodine, (±)-carbodine and ribavirin against various H1N1, H3N2 and influenza type B strains

Virus type	Inoculum[b]	Compounds	Anti-influenza activity (H1N1, H3N2 & FluB)[a]						
			EC_{50} [c] (μM)		EC_{90} [d] (μM)	IC_{50} [e] (μM)	SI[f]		
			Visual CPE	Neutral Red	Virus Yield Reduction		Visual CPE	Neutral Red	Virus Yield Reduction
A/California/07/2009/H1N1 (swine flu strain)	100	(−)-Carbodine	1.3 ± 0.20	1.6 ± 0.3	3.8 ± 0.3	>414	>318	>259	>109
		(+)-Carbodine	>414	>414	>414	>414	1	1	1
		(±)-Carbodine	1.5 ± 1.0	1.9 ± 1.0	4.4 ± 2.3	>414	>276	>218	>94
		Ribavirin	11.2 ± 6.3	19.4 ± 1.8	13.0 ± 4.2	>1310	>117	>68	>101
A/Solomon Island/03/2006/H1N1	21	(−)-Carbodine	0.7 ± 0.4	0.6 ± 0.3	1.3 ± 0.9	>414	>591	>690	>318
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	1.9 ± 0.5	2.0 ± 0.4	2.3 ± 0.4	>414	>218	>207	>180
		Ribavirin	23.3 ± 0.7	25.1 ± 3.6	27.0 ± 6.4	>1310	>56	>52	>49
A/Wisconsin/67/ 2005/H3N2	10	(−)-Carbodine	1.6 ± 0.7	2.0 ± 0.9	2.5 ± 0.4	>414	>259	>207	>166
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	2.8 ± 0.7	4.6 ± 3.9	3.5 ± 1.1	>414	>148	>90	>118
		Ribavirin	16.4 ± 7.2	20.2 ± 0.6	21.7 ± 11.9	>1310	>80	>65	>60
B/Malaysia/ 2506/2004	27	(−)-Carbodine	0.6 ± 0.2	0.8 ± 0.1	1.5 ± 1.0	>414	>690	>518	>276
		(+)-Carbodine	>41	>41	>41	>414	<10	<10	<10
		(±)-Carbodine	0.8 ± 0.0	1.3 ± 0.3	3.8 ± 0.9	>414	>518	>318	>109
		Ribavirin	10.2 ± 5.6	19.5 ± 5.6	22.2 ± 1.9	>1310	>128	>67	>59

[a] Average of triplicate experimental data.
[b] Approximate $CCID_{50}$ inoculated per well; same inoculum used for all replicate tests.
[c] Effective concentration required to reduce influenza virus-induced cytopathic effect by 50%.
[d] Concentration required to reduce infectious virus titer by 90%.
[e] Concentration of drug for 50% cell inhibition without virus.
[f] In vitro selectivity index (IC_{50}/EC_{50}) and for virus yield reduction (IC_{50}/EC_{90}).

pound **9** in good yield. Finally, the compound **9** was converted to a cytosine using a standard reaction condition followed by removal of protecting groups with TFA/H_2O afford (−)-carbodine **1**[11] in good yield (overall 35% yield from intermediate **4**).

The anti-influenza activity of (−)-carbodine was evaluated in vitro in comparison to (+)-carbodine, (±)-carbodine and ribavirin, and it showed significant anti-influenza activity (Table 1). Virus strains tested were Influenza A/Duck/MN/1525/81/H5N1 and A/Gull/PA/4175/83/H5N1 obtained from R. Webster of St. Jude Children's Research Hospital (Memphis, TN) and A/Vietnam/1203/2004H/H5N1 and A/Hong Kong/213/2003H/H5N1, hybrids of Ann Arbor/6/60 containing the Asian H5 and N1 segments and provided by MedImmune. A/California/07/2009/H1N1 (swine flu strain), A/Solomon Islands/03/2006/H1N1, A/Wisconsin/67/2005/H3N2, and B/Malaysia/2506/2004 were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, Ga.). Triplicate wells of Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34) were treated with serial dilutions of the test compounds in 96-well plates then inoculated with $\leqslant$300 $CCID_{50}$ of virus, sufficient to induce ~100% cytopathic effect (CPE) in control wells within three days. The antiviral activity of test compounds was measured by inhibition of virus-induced CPE,[12] neutral red (NR) dye uptake,[13] and virus yield reduction assays,[14] essentially as described previously. For comparison, (±)-carbodine (SRI 5670 lot 11-30-2000, provided by Southern Research Institute through the NIAID Antiviral Substances Program), and ribavirin (obtained from ICN Pharmaceuticals in Costa Mesa, CA), were tested in parallel for each of the triplicate assays (duplicate for Hong Kong strain). Carbodine samples were diluted in minimal essential medium, purified water, or DMSO and held at −20 °C until tested, and ribavirin was diluted from powder for each replicate assay.

The anti-influenza in vitro activity of (−)-carbodine, (+)-carbodine, (±)-carbodine and ribavirin against various H5N1 strains is shown in Table 1. In visual CPE and neutral red assays, the anti-influenza in vitro activity of (−)-carbodine (0.7–2.0 μM) was more potent than that of (±)-carbodine (0.7–5.0 μM) against the H5N1 strains, while (+)-carbodine was devoid of antiviral activity. In virus yield reduction assays, (−)-carbodine was about twofold more active than (±)-carbodine with the Duck and Vietnam strains, and equally potent as (±)-carbodine with Gull and Hong Kong strains.

Table 2 shows the in vitro anti-influenza activity of (−)-carbodine, (+)-carbodine, (±)-carbodine and ribavirin against novel 2009 H1N1, H1N1, H3N2 and influenza type B strains. In A/California/07/2009/H1N1 strain (swine flu strain), (−)-carbodine showed an EC_{50} value of 1.3 μM in the visual CPE assay, an EC_{50} of 1.6 μM in neutral red assay and an EC_{90} of 3.8 μM in virus yield reduction assay with selectivity indexes of >318, >259 and >109, respectively. The (−)-carbodine was consistently more active than the other compounds against all four virus strains in Table 2. In A/Solomon Island/03/2006/H1N1 strain, (−)-carbodine was 2 to 3 times more potent than (±)-carbodine and >20-fold more potent than ribavirin with high selectivity indices in visual CPE as well as in the neutral red assay. The (−)-carbodine was slightly more active than (±)-carbodine and ribavirin in all assays against the A/Wisconsin/67/2005/H3N2 strain. In influenza type B (Malaysia/2506/2004 strain), (−)-carbodine was relatively more active than (±)-carbodine, 15–24-fold more potent than ribavirin in visual CPE, neutral red and virus yield reduction assays.

In summary, (−)-carbodine was shown to be the active form of (±)-carbodine as an anti-influenza agent. The (±)-carbodine racemic mixture showed less activity than (−)-carbodine, as expected, but the differences were not significant by Mann–Whitney analysis (P >0.05), and further assays with fresh (±)-carbodine may be merited for this comparison. The (−)-carbodine was identified as a potent anti-influenza agent against in vitro A/Duck/MN/1525/81/H5N1, A/Gull/PA/4175/83/H5N1, A/Hong Kong/213/2003H/H5N1 and A/Vietnam/1203/2004H/H5N1 strains, which was further confirmed by virus yield reduction assay. Moreover, (−)-carbodine showed potent activity against novel H1N1 (A/California/07/2009/H1N1) (swine), A/Solomon Island/03/2006/H1N1, A/Wisconsin/67/2005/H3N2 and B/Malaysia/2506/2004.

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11. *Data of compound* **1**:[5e] White solid. Mp 214 °C; $[\alpha]_D^{27}$ −74.5 (c 0.52, H_2O); UV (H_2O) λ_{max} 275.0 nm (pH 7), 285 (pH 2), 275 (pH 11); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.23 (m, 1H), 1.94 (m, 1H), 2.01 (m, 1H), 3.42 (m, 2H), 3.80 (dd, J = 4.5, 9.0 Hz, 1H), 4.01 (m, 1H), 4.54 (d, J = 4.5 Hz, 1H), 4.59 (q, 1H), 4.65 (t, J = 5.0 Hz, 1H), 4.72 (d, J = 7 Hz, 1H), 5.69 (d, J = 7.5 Hz, 1H), 6.96 (br s, 1H), 7.01 (br s, 1H), 7.60 (d, J = 7.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7, 156.6, 143.8, 93.8, 73.8, 72.1, 63.4, 61.7, 45.4, 29.0. Anal. Calcd for $C_{10}H_{15}N_3O_4$: C, 49.77; H, 6.27; N, 17.42. Found: C, 49.67; H, 6.31; N, 17.43.
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