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Design of inhibitors of influenza virus membrane fusion: Synthesis, structure–activity relationship and *in vitro* antiviral activity of a novel indole series ☆

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ABSTRACT

The fusion of virus and endosome membranes is an essential early stage in influenza virus infection. The low pH-induced conformational change which promotes the fusogenic activity of the haemagglutinin (HA) is thus an attractive target as an antiviral strategy. The anti-influenza drug Arbidol is representative of a class of antivirals which inhibits HA-mediated membrane fusion by increasing the acid stability of the HA. In this study two series of indole derivatives structurally related to Arbidol were designed and synthesized to further probe the foundation of its antiviral activity and develop the basis for a structure–activity relationship (SAR). Ethyl 5-(hydroxymethyl)-1-methyl-2-(phenysulphanylmethyl)-1*H*-indole-3-carboxylate **(15)** was identified as one of the most potent inhibitors and more potent than Arbidol against certain subtypes of influenza A viruses. In particular, **15** exhibited a much greater affinity and preference for binding group 2 than group 1 HAs, and exerted a greater stabilising effect, in contrast to Arbidol. The results provide the basis for more detailed SAR studies of Arbidol binding to HA; however, the greater affinity for binding HA was not reflected in a comparable increase in antiviral activity of **15**, apparently reflecting the complex nature of the antiviral activity of Arbidol and its derivatives.

1. Introduction

Influenza is one of the most infectious diseases and is responsible for very significant morbidity and mortality annually around the world. The viruses belong to the *Orthomyxoviridae* family; they have a segmented, single-stranded, negative-sense RNA genome and are divided into three genera A, B and C, according to their internal components (Palese and Shaw, 2007). The influenza A viruses are classified by subtype, based on the antigenic characteristics of the two surface glycoproteins embedded in the lipid envelope of the virion, the haemagglutinin (HA) and the neuraminidase (NA). Sixteen subtypes of HA and nine of NA result in the existence of a large number of virus subtypes with different combinations of HA and NA, only a few of which, currently H1N1 and H3N2, have circulated in the human population (Wright et al., 2007). Most influenza A viruses are maintained in a vast natural reservoir in wild waterfowl and shorebirds, from which they may emerge to cause disease in domestic animals and humans; influenza B and C viruses principally infect humans.

Vaccination is the mainstay of control and prevention strategies against influenza and is based on the generation of neutralizing antibodies against the HA. However, antigenic drift in the HA necessitates frequent change in vaccine composition, and the time (6–9 months) involved in producing and delivering vaccines against newly emergent variants can impact the effectiveness of this approach (Ampofo et al., 2012). Anti-influenza drugs complement vaccines and their use is particularly important in combating the initial stages of an emergent pandemic, when

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vaccines against the novel virus are not available (Hayden and Pavia, 2006).

The widely available influenza antivirals are currently limited to two classes of agents: the neuraminidase inhibitors (oseltamivir and zanamivir) and the M2 inhibitors (amantadine and rimantadine) (Moscona, 2005). However, their effectiveness has been limited by the emergence of drug-resistant viruses. This is especially so for the M2 inhibitors, in that the seasonal A(H3N2) viruses have recently acquired resistance and the seasonal A(H1N1) viruses were replaced by the amantadine-resistant pandemic H1N1 2009 viruses (Deyde et al., 2007; Hay et al., 2008; Gubareva et al., 2009). A significant increase in amantadine resistance among some highly pathogenic H5N1 viruses have further limited the potential usefulness of these antivirals (Hurt et al., 2007).

On the other hand, neuraminidase inhibitor drugs have been favoured clinically, since they are effective against influenza B as well as all influenza A NA subtypes, are well tolerated, and appear to have a higher barrier to resistance emergence (Moscona, 2005). However, the previous seasonal A(H1N1) viruses, circulating in 2007–2009 acquired oseltamivir-resistance (Collins et al., 2009) and oseltamivir-resistant variants have occurred sporadically among the novel A(H1N1)pdm09 viruses that emerged in April 2009 (Hurt et al., 2011); and oseltamivir is the therapeutic agent favoured by clinicians (Bautista et al., 2010; Ling et al., 2010). There is therefore an urgent need for more antivirals against influenza; the only other licensed drugs with anti-influenza activity are ribavirin, which has been used to only a very limited extent to treat severe infections, and Arbidol which is marketed in Russia and China and a few other countries (Boriskin et al., 2008).

Potential novel targets being explored for the development of new anti-influenza agents include the viral polymerase (and endonuclease), the non-structural protein NS1 and the HA (Das et al., 2010). Blocking virus entry into the host cell (Luo, 2012) is already an effective strategy, as it is the target of the M2 inhibitors and is the basis of vaccination. The trimeric envelope glycoprotein HA plays a key role in promoting fusion between the virus and endosome membranes during virion internalization by endocytosis, as well as being responsible for attachment to sialylglycan receptors on the cell surface (Skehel and Wiley, 2002). The protein contains two disulfide-linked polypeptide chains, HA1 and HA2; the HA1 subunit contains the receptor binding site and HA2 the N-terminal fusion peptide. The acidic pH in the endosome triggers a conformational change in the HA whereby the fusion peptide is inserted into the target membrane promoting fusion between the virus and endosome membranes. Prior to this event passage of protons through the M2 channel causes acid-induced dissociation of the internal ribonucleoprotein (RNP) – matrix (M1) structure to effect release of the virus RNP into the cytosol for transport into the nucleus to initiate replication (Hay et al., 2008) Cloroquine and high concentrations of amantadine, like other acidotropic agents, can inhibit non-specifically virus infection *in vitro* by increasing endosome pH and preventing the low pH-dependent structural transition (Di Trani et al., 2007; Daniels et al., 1985).

Several inhibitors of influenza replication *in vitro*, including Arbidol, have been shown to specifically target the fusion-mediating conformational change in HA by increasing the acid stability of the protein (Skehel and Wiley, 2002). However, many of them are subtype specific. For example, a series of benzo- and hydro-quinones, including *tert*-butyl hydroquinone (TBHQ), were shown to bind in a pocket of the H3 (group 2) HA and prevent the native HA from undergoing the low pH-induced conformational change, but not to bind H1 or H2 (group 1) HAs (Bodian et al., 1993). In contrast, a number of other inhibitors, including a quinolizidine-linked benzamide, were shown to block the HA conformational change of H1 and H2, but not of H3, subtypes (Luo et al., 1997; Plotch et al., 1999). More recently, a new class of N-(1-thia-4-azaspiro[4.5]decan-4-yl)carboxamide inhibitors that also show specific activity against H3 subtype viruses was reported (Vanderlinden et al., 2010; Zhan et al., 2012). Arbidol, on the other hand, has been shown to be more broadly effective in inhibiting different influenza A subtypes (with both group 1 and 2 HAs) and influenza B viruses (Boriskin et al., 2008).

The basis of the antiviral efficacy of Arbidol is, however, unclear since it has proven to be effective in the treatment of several other respiratory viral infections in addition to influenza and it inhibits the replication *in vitro* of a variety of enveloped and non-enveloped viruses, (Boriskin et al., 2008) including, for example, respiratory syncytial virus, parainfluenza virus, rhinovirus, and hepatitis B and C viruses (Brooks et al., 2004; Chai et al., 2006; Pécheur et al., 2007). This broad spectrum of activity has complicated interpretation of the molecular basis of Arbidol action, for example, the relative importance of interaction with the lipid membrane or with protein components involved in the membrane fusion process. (Teissier et al., 2011). However, Leneva et al. (2009) have shown that in the case of influenza, properties of the HA, in particular the pH of fusion, are major determinants of the sensitivity of virus replication *in vitro*, indicating that Arbidol inhibits virus entry by interacting directly with HA to stabilize it against the low pH-induced conformational change mediating membrane fusion.

With the aim of identifying novel lead compounds active against emergent human infectious diseases (Perfetto et al., 2013; Peduto et al., 2011) and to gain a better understanding of the structural features of Arbidol important for its (broader) antiviral activity and HA binding properties, and the relationship between HA binding and antiviral activity, in this paper we report the design, synthesis and structure–activity (SAR) studies of several new ethyl 1*H*-indole-3-carboxylate derivatives structurally related to Arbidol. Retaining the indole skeleton, modifications were introduced in positions 2, 4, 5 and 6 of the heterocyclic ring. The compounds synthesized can be classified into two different series (Chart 1). The first series (Series I) includes alcohol derivatives (**6, 7, 15, 16, 18** and **21**) which lack an amino substituent in position 4 (Table 1A). The second series (Series II) comprises compounds **24, 28a–h, 29a–m**, **30, 31** and **32** in which the hydroxy group in position 5 was replaced by different amino substituents (Chart 1, Table 1B).

Compounds were tested against a range of influenza viruses. Of all the compounds, ethyl 5-(hydroxymethyl)-1-methyl-2-(phenysulphanylmethyl)-1*H*-indole-3-carboxylate (**15**) was identified to be one of the most potent inhibitors, with a therapeutic index greater than Arbidol for most viruses tested. To investigate the basis of its improved activity, we compared the effects of **15** with those of Arbidol on HA-mediated membrane fusion, assayed by haemolysis and heterokaryon formation, and their interaction with HA, in fluorescence quenching and thermal denaturation assays. While the effects of **15** were somewhat greater than those of Arbidol against membrane fusion (and virus replication), the affinity of binding of **15** to HA was substantially higher and in particular exhibited a much greater preferential binding to group 2 than to group 1 HAs.

2. Materials and methods

2.1. Chemical synthesis of the compounds

All reagents were analytical grade and purchased from Sigma–Aldrich (Milano, Italy). Flash chromatography was performed on Carlo Erba silica gel 60 (230–400 mesh; CarloErba, Milan, Italy). TLC was carried out using plates coated with silica gel 60 F 254 nm purchased from Merck (Darmstadt, Germany). ^{1}H NMR

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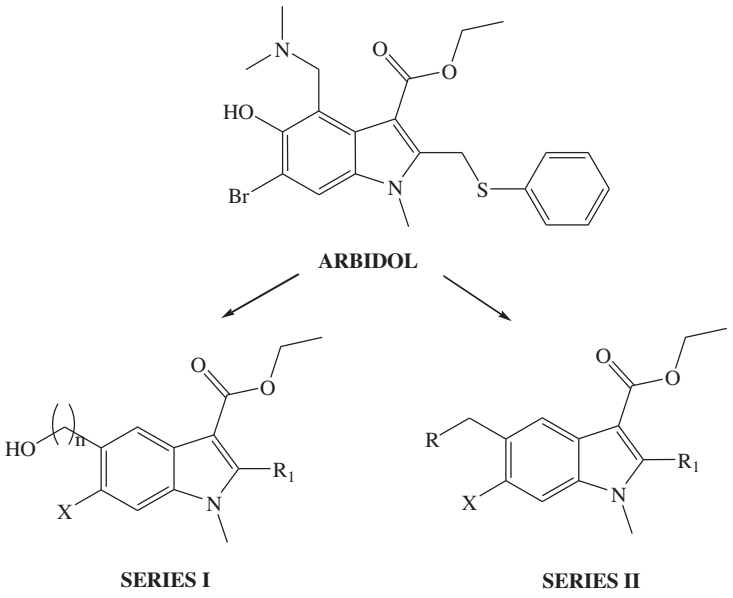
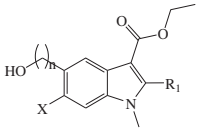


Chart 1. Chemical structures of Series I and II compounds.

Table 1A
Inhibition of the replication of different influenza viruses by Arbidol and Series I compounds.

Cpds			Virus tested IC_{50} (μM)									
	X	*n*	R_1	H1N1[a]	H1N1[b]	H1N1pdm[c]	H3N2[d]	H3N2[e]	H4N6[f]	H7N3[g]	H8N4[h]	H11N9[i]	B[j]
6	H	0	CH_2SPh	31.3 (5.6)	62.5 (4.5)	31.3 (2.3)	31.3 (1.3)	31.3 (2.3)	62.5 (2.8)	–	–	62.3 (2.6)	–
7	Br	0	CH_2SPh	31.3 (2.2)	125 (4.9)	15.6 (1.7)	62.5 (3.5)	125 (2.3)	31.3 (0.5)	–	–	125 (3.0)	–
15	H	1	CH_2SPh	15.6 (5.2)	31.3 (3.0)	15.6 (1.9)	15.6 (3.1)	3.9 (2.3)	7.8 (2.8)	125 (2.3)	31.3 (1.6)	31.3(2.5)	62.5 (2.5)
16	H	1	CH_3	–	–	–	–	–	125 (2.9)	–	–	125 (3.5)	–
18	Br	1	CH_3	7.9 (3.8)	125 (2.0)	7.9 (1.8)	–	–	7.9 (2.1)	31.3 (1.9)	7.9 (2.3)	7.9 (1.9)	–
21	Br	1	CH_2SPh	–	–	–	–	62.5 (2.4)	125 (2.5)	–	125 (2.5)	125 (2.5)	–
ARB		11.8 (4.4)	15.6 (3.1)	23.4 (1.3)	15.6 (1.7)	7.8 (2.5)	31.3 (2.3)	15.6 (1.3)	15.6 (1.7)	–	31.3 (1.9)		

A dash indicates no effect at 125 μM compound. Standard deviations are in parenthesis.
[a] A/Brisbane/59/07.
[b] A/PR/8/34.
[c] A/California/7/09.
[d] A/Wuhan/359/95.
[e] A/X31.
[f] A/dk/Czechoslovakia/56.
[g] A/ty/Italy/02.
[h] A/ty/Ontario/68.
[i] A/dk/Memphis/74.
[j] B/Brisbane/60/06.

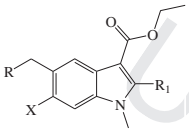
spectra were registered on a Brucker AC 300. Chemical shifts are reported in ppm. The abbreviations used are follows: s, singlet; d, doublet; dd double doublet; bs, broad signal. Mass spectrometry analysis ESI-MS was carried out on a Finnigan LCQ Deca ion trap instrument.

*2.1.1. General synthesis of Series I (**6, 7, 15, 16, 18** and **21**)*

The title compounds, ethyl-5-hydroxy or 5-hydroxymethyl-2-methyl-1*H*-indole-3-carboxylate derivatives were obtained as described in Schemes 1–3 (Supporting information). The synthesis of 5-hydroxyindole derivatives **6** and **7** started from commercially available ethyl 5-hydroxy-2-methylindole-3-carboxylate (**1**), which underwent a protection reaction using acetic anhydride and pyridine, affording intermediate **2** in good yield. Methylation with iodomethane under basic condition (Cao et al., 2005) in DMF provided compound **3** which was submitted to radical bromination yielding a mixture of mono and dibromo derivatives **4** and **5**, easily separated by chromatography on silica gel. Final nucleophilic displacement by thiophenol (Trofimov et al., 1993) gave the desired compounds **6** and **7** (Scheme 1).

Compound **8**, prepared as described in the literature, (Weinstain et al., 2008) was the common intermediate for the synthesis of 5-hydroxymethyl-indole derivatives **15**, **16**, **18** and **21**. It was reacted with two different β-ketoesters **9** and **10** in a copper-catalyzed Ullmann-type coupling reaction (Sellitto et al., 2010; Tanimori et al., 2007) giving the key intermediates **11** and **12**. Subsequent N-alkylation with iodomethane using Kikugawa's procedure afforded compounds **13** and **14** in quantitative yields.

Table 1B
Inhibition of the replication of different influenza viruses by Series II compounds.

Cpds				Virus tested IC_{50} (μM)									
	X	R_1	*R*	H1N1[a]	H1N1[b]	H1N1pdm[c]	H3N2[d]	H3N2[e]	H4N6[f]	H7N3[g]	H8N4[h]	H11N9[i]	B[j]
24	H	CH_3	NH_2	–	–	–	–	–	–	–	–	–	–
28a	H	CH_2SPh	$N(CH_3)_2$	7.9 (0.8)	15.6 (2.8)	7.9 (0.2)	7.9 (0.2)	3.9 (0.5)	7.9 (0.2)	31.3 (2.9)	7.9 (1.3)	7.9 (0.2)	31.3 (1.4)
28b	H	CH_2SPh	$N(CH_2CH_3)_2$	3.9 (0.7)	15.6 (1.3)	3.9 (0.5)	7.9 (1.2)	3.9 (0.4)	7.9 (0.7)	31.3 (2.7)	7.9 (1.0)	7.9 (0.4)	31.3 (1.0)
28c	H	CH_2SPh	$N(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$	3.9 (0.4)	3.5 (0.4)	3.9 (0.7)	7.9 (0.4)	15.6 (1.1)	7.9 (0.6)	7.9 (0.9)	7.9 (1.3)	7.9 (0.7)	15.6 (0.2)
28d	H	CH_2SPh	Pyrrolidine	7.9 (0.9)	31.3 (2.6)	7.9 (1.1)	7.9 (1.0)	15.6 (0.9)	7.9 (0.9)	46.9 (2.5)	15.6 (1.4)	7.9 (0.8)	125 (1.2)
28e	H	CH_2SPh	N-methyl piperazine	7.9 (0.3)	31.3 (1.0)	7.9 (0.8)	7.9 (0.7)	15.6 (0.5)	7.9 (0.3)	31.3 (1.8)	15.6 (1.3)	7.9 (1.2)	–
28f	H	CH_2SPh	NHBoc-piperazine	31.3 (2.3)		31.3 (1.9)		62.5 (2.0)					
28g	H	CH_2SPh	N-(2,4 difluoro)phenylpiperazine	31.3 (3.3)		31.3 (0.3)		62.5 (0.6)					
28h	H	CH_2SPh	Morpholine	31.3 (0.7)		31.3 (1.8)		62.5 (1.4)					
29a	H	CH_3	$N(CH_3)_2$	125 (2.0)	62.5 (1.7)	–	125 (2.3)	–	–	–	–	–	–
29b	H	CH_3	$N(CH_2CH_3)_2$	62.5 (1.4)	125 (1.2)	125 (1.7)	15.6 (1.2)	125 (2.9)	–	–	125 (0.8)	125 (1.6)	125 (1.8)
29c	H	CH_3	$N(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$	7.9 (0.6)	7.9 (0.8)	7.9 (0.6)	15.6 (1.4)	7.9 (0.5)	15.6 (1.9)	15.6 (1.8)	31.3 (0.8)	15.6 (1.7)	31.3 (1.6)
29d	H	CH_3	Pyrrolidine	31.3 (0.7)	–	31.3 (0.7)	31.3 (0.7)	62.5 (0.9)	125 (1.7)	125 (1.6)	–	–	–
29e	H	CH_3	*N*-methyl-piperazine	31.3 (0.6)	–	31.3 (1.0)	31.3 (1.3)	62.5 (1.3)	125 (1.7)	125 (2.1)	–	–	–
29f	H	CH_3	2-piperazin-1-ylethylamino	31.3 (1.6)		31.3 (0.5)		62.5 (1.2)					
29g	H	CH_3	$N(CH_3)CH_2CH_2N(CH_2CH_3)_2$	31.3 (0.4)		31.3 (0.5)		–					
29h	H	CH_3	$N(CH_2CH_3)CH_2CH_2N(CH_2CH_3)_2$	31.3 (3.0)		31.3 (0.9)		–					
29i	H	CH_3	$NHCH_2CH_2NHCH_3$	31.3 (1.2)		31.3 (1.2)		62.5 (1.2)					
29j	H	CH_3	$NHCH_2CH_2NHBoc$	31.3 (0.7)		31.3 (0.6)		62.5 (1.7)					
29k	H	CH_3	$NHCH_2CH_2N(CH_3)_2$	31.3 (1.8)		31.3 (0.8)		62.5 (0.7)					
29l	H	CH_3	$NHCH_2CH_2OH$	31.3 (2.3)		31.3 (0.4)		62.5 (0.8)					
29m	H	CH_3	$NHCH_2CH_2NHCH_2CH_2OH$	31.3 (0.9)		31.3 (2.2)		15.6 (1.3)					
30	Br	CH_3	$N(CH_3)_2$	62.5 (1.0)	125 (1.9)	125 (1.2)	15.6(2.0)	–	–	–	125 (1.3)	125 (1.3)	125 (1.5)
31	H	CH_3	Diazepan	–	125 (2.8)	–	–	–	–	125 (1.8)	–	–	–
32	H	CH_2SPh	*N*-methyl-pyrrolidinium iodide	31.3 (0.5)		31.3 (0.4)		62.5 (0.8)					

A dash indicates no effect at 125 μM compound.
[a] A/Brisbane/59/07.
[b] A/PR/8/34.
[c] A/California/7/09.
[d] A/Wuhan/359/95.
[e] A/X31.
[f] A/dk/Czechoslovakia/56.
[g] A/ty/Italy/02.
[h] A/ty/Ontario/68.
[i] A/dk/Memphis/74.
[j] B/Brisbane/60/06.

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Final deprotection using TBAF in THF (Corey et al., 1977) furnished the 5-hydroxymethyl derivatives **15** and **16** in excellent yields (Scheme 2). Treatment of **14** with 1 equiv. of NBS (Trofimov et al., 1993) allowed us to obtain monobromo **17** which was further deprotected, as previously described, to give compound **18**. Using 2 equiv. of NBS, intermediate **14** underwent dibromination in positions 2 and 6 and conversion of the hydroxy-methyl group in position 5 into aldehyde to yield **19**. Reduction with $NaBH_4$ and subsequent treatment with thiophenol in EtOH permitted us to recover compound **21** (Scheme 3).

2.1.2. General synthesis of Series II (*24, 28a–h, 29a–n, 30, 31, 32*)

The title compounds, ethyl-5-aminomethyl-1-methyl-1*H*-indole-3-carboxylate derivatives were obtained as described in Schemes 4–6 (Supporting information). The synthesis of primary amine **24** is described in Scheme 4. Ethyl 1,2-dimethyl-5-(hydroxymethyl)-1*H*-indole-3-carboxylate (**16**) was treated with PPh_3 in a mixture of CCl_4 and DMF at room temperature, affording the corresponding chloromethyl derivative **22** (Hübner et al., 2000). Gabriel synthesis and subsequent hydrazinolysis furnished ethyl 5-(aminomethyl)-1,2-dimethyl-1*H*-indole-3-carboxylate **24** in good yield (Roubini et al., 1991).

Amines **28a–h**, **29 a–n** and **30** were synthesized according to the procedure described in Scheme 5 (Supporting information). Oxidation of alcohols **15**, **16** and **18** with pyridinium dichromate led to the formation of aldehydes **25**–**27** in high yields. Subsequent reductive amination with several aliphatic amines, using $NaBH(OAc)_3$ as reducing agent, provided the desired compounds **28a–h**, **29a–n** and **30** in excellent yields. Ethyl 5-(1,4-diazepan-1-ylmethyl)-1,2-dimethyl-indole-3-carboxylate **31** was synthesized from Boc deprotection of amine **29n** using TFA in CH_2Cl_2 (Schemes 6 in Supporting information). Conversion of ethyl 1-methyl-2-(phenylsulfanylmethyl)-5-(pyrrolidin-1-ylmethyl)-1*H*-indole-3-carboxylate **29d** into a quaternary ammonium salt **32** was performed using methyl iodide in ethyl acetate.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle Medium (Sigma D6429), supplemented with 10% foetal calf serum (FCS) inactivated at 56 °C for 1 h, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma P0781), in a humidified atmosphere with 5% CO_2.

The influenza viruses were from stocks held by the WHO Collaborating Centre for Reference and Research on Influenza, at NIMR: A/Puerto Rico/8/34(H1N1) (PR8), A/Brisbane/59/2007(H1N1), A/California/7/2009(H1N1pdm09), A/Singapore/1/57(H2N2), A/Wuhan/359/95(H3N2), recombinant virus X31(H3N2) (A/Aichi/2/68 × PR8), A/duck/Czechoslovakia/56(H4N6), A/turkey/Italy/214845/2002(H7N3), A/turkey/Ontario/6118/68(H8N4), A/duck/Memphis/546/74(H11N9), B/Brisbane/60/2006, and four mutants of X31(H3N2), three with increased pH of fusion, 1a (HA2 D112G), 2a (HA2 R54K) and ab4 (HA1 H17R), resistant to high concentrations of amantadine (Daniels et al., 1985), and the antibody escape mutant V9A (HA1 G218R) (Daniels et al., 1987). The attenuated recombinant virus RG14(H5N1) (A/Vietnam/1194/2004 × PR8) was obtained from the National Institute for Biological Standards and Control. Virus stocks were grown in the allantoic cavities of 11-day-old embryonated hen eggs for use in all the experiments. The allantoic fluid was clarified by low-speed centrifugation and the virus titre determined by haemagglutination (HA) assay, and stored at −80 °C.

2.3. Evaluation of cell viability by neutral red assay

The neutral red assay determines the accumulation of the neutral red dye in the lysosomes of viable cells after incubation with test agent. MDCK cells were seeded in 96-well plates and incubated for 24 h in a humidified atmosphere at 5% CO_2. The cells were exposed to twofold serial dilutions of compound, dissolved in DMEM with 5% FCS, ranging from 250 μM to 3.9 μM, and incubated for 24 h and 48 h. Cells were then washed with phosphate buffered saline (PBS) and incubated for 2 h with neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride-Sigma, N4638) dissolved in serum-free DMEM at a final concentration of 40 μg/ml. Cells were washed again with PBS to remove the dead cells and the dye was extracted from the intact cells in 1% glacial acetic acid in 50% ethanol (Glacial acetic acid, Sigma, 537020; Ethanol, Riedel-de Haen, 32294). The fluorescence was read in a spectrofluorimeter with excitation and emission wavelengths of 530 nm and 645 nm, respectively. Three independent experiments were performed and the CC_{50} (drug concentration required to reduce cell viability by 50%) was calculated at 24 h and 48 h.

2.4. Plaque reduction assay

The virus plaque assay, using low-viscosity overlay medium in a 96-well format, (Matrosovich et al., 2006) was used to measure the inhibition of virus replication by Arbidol and its analogues. Briefly, MDCK cells 3×10^3 cells per well were seeded in 96-well plates in DMEM, containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and were incubated at 37 °C until 90% confluence. Twofold serial dilutions of each compound from 125 μM to 3.9 μM were added to duplicate wells. After incubation for 30 min at 37 °C, 100 μl of virus-containing allantoic fluid (approximately 0.1 PFU/cell) was added to each well, except uninfected control cells. Each microtitre plate included uninfected control wells, virus-infected control wells and virus-infected wells containing compound. After 3 h of infection, cells were overlaid with an equal volume of DMEM medium supplemented with 0.2% Bovine Serum Albumin, 1.2% (w/v) Avicel, and 2.5 μg/ml TPCK-trypsin (Sigma T-1426).

After 24 h incubation at 37 °C in a humidified atmosphere with 5% CO_2, cells were washed and fixed by adding 50 μl of cold 4% paraformaldehyde in PBS. Cells were incubated for 1 h with a mouse monoclonal antibody against influenza type A or B nucleoprotein and then with a peroxidase-labelled anti-mouse antibody, diluted 1:4000 and 1:1000, respectively, in ELISA buffer, containing 10% horse serum, 0.1% Tween-80 in PBS. Cells were then incubated with True Blue™ substrate (KPL, Gaithersburg, USA), 0.03% H_2O_2, until colour developed. Plaques were counted and the IC_{50} value (concentration of compound required to reduce the number/size of plaques by 50%) was calculated (Sullivan et al., 2012). Three independent experiments were performed.

2.5. Haemolysis inhibition assay

Virus-induced haemolysis was estimated as described previously using human erythrocytes (Wharton et al., 1994; Steinhauer et al., 1995). Wild type X31 and mutants 1a, 2a, ab4 and V9A were used. Each influenza virus was added to 1 ml 1% human red blood cells (RBC) in PBS and was incubated at 37 °C in the presence or absence of 40 μM Arbidol or **15**. The pH was then varied over the range 4.6 to 7.0 using citrate buffer (0.15 M sodium citrate pH 3.5). After 30 min incubation, cell debris and un-lysed cells were removed by centrifugation at 2000*g* for 10 min and the absorbance of the released haemoglobin in the supernatant was read at 520 nm.

2.6. Heterokaryon assay of fusion between cells expressing X31 HA

Chinese Hamster Ovary (CHO) cells transfected with the X31 HA gene (Godley et al., 1992) were grown in 24-well plates. Confluent

monolayers were treated with TPCK-treated trypsin for 5 min at 37 °C, in order to the cleave HA0 to the fusion active HA1/HA2, and then with 40 μM **15** or Arbidol for 30 min at 37 °C. Cells were then incubated with citrate buffer at different pH values and heterokaryon formation was monitored microscopically every 10 min.

2.7. Purification of HA

The soluble ectodomain of HA (BHA) was released from purified virus, grown in hen eggs, by incubation with the protease bromelain (1:10 (w/w) bromelain:virus) for 16 h at 37 °C in 0.1 M Tris/HCl pH 7.5, 50 mM 2-mercaptoethanol, and was purified by sucrose gradient sedimentation and ion exchange chromatography as previously described (Ha et al., 2002).

2.8. Tryptophan fluorescence assay

Increasing amounts of **15** or Arbidol (from 5 mM stock solutions in DMSO) were added to BHA (0.2 μM in PBS pH 6.5) at 20 °C and tryptophan fluorescence was measured using a Jasco FP-6300 spectrofluorimeter with excitation at 285 nm and emission at 340 nm. Fluorescence intensities were corrected for inner filter effects and equilibrium dissociation constants (K_d) were determined by non-linear least-squares fitting to a one-site binding model.

2.9. Thermal shift assay

The assay was performed as previously described (Niesen et al., 2007). BHA (0.4 μM in PBS pH 6.5) was incubated with **15** or Arbidol (final concentration 50 μM, 1% DMSO), or with1% DMSO, for 15 min at 25 °C. SYPRO Orange (Sigma; 5X) was added and the temperature of the preparation was increased at 5 °C per min, and the fluorescence measured every 2 °C increase (excitation 470 nm, emission 570 nm) using a Jasco FP-6300 instrument. The T_m was estimated as the temperature at the midpoint of the change in SYPRO fluorescence brought about by thermal unfolding of the BHA.

3. Results

3.1. Inhibition of influenza virus replication by 1H -indole-3-carboxylate derivatives

Arbidol and its derivatives were tested at different concentrations (from 3.9 μM to 125 μM) for their ability to inhibit influenza virus replication in MDCK cells, in a plaque reduction assay. Different types and subtypes of influenza viruses were used initially: three different strains of subtype A(H1N1) and two of subtype A(H3N2), and single strains of subtypes H4N6, H7N3, H8N4 and H11N9, and a type B virus. Differences were observed in the sensitivities of the different viruses to particular derivatives as well as in the effectiveness of different compounds, expressed as IC_{50} (Tables 1A and 1B). In later tests, only three viruses, two H1N1 and one H3N2 subtype A viruses, were used, those which had shown higher susceptibility to inhibition.

The cytotoxicities of the compounds (from 3.9 μM to 250 μM) in MDCK cells were assessed at 24 and 48 h using a neutral red viability assay. The results are expressed as the CC_{50} (the concentration of compound causing 50% reduction in cell viability) (Table 2). No toxicity was observed with the alcohol derivatives (Series I) at concentrations below 250 μM, except for compound **7** that was slightly cytotoxic. In contrast, as regards the compounds of Series II, most with a thiophenyl ring in position 2 (**28a–e**) were highly cytotoxic**.** The toxicity disappeared when a positive charge was inserted into the amino substituent (**32** *vs* **28d**). The replacement of thiophenyl by a methyl in position 2 of the indole ring reduced significantly the toxicity (**24, 29a–b, 29d–m**, **30** and **31**), except for compound **29c** which retained toxicity similar to its analogue **28c**, demonstrating the high cytotoxic potential of this particular amine.

Table 2
Cytotoxicities of Arbidol and its derivatives in MDCK cells.

CC50 μM					
Compounds	24 h	48 h	Compounds	24 h	48 h
Arbidol	115.2	117	29a	LC	LC
6	LC[a]	LC	29b	LC	LC
7	125	31.3	29c	52.0	49.7
15	LC	LC	29d	LC	LC
16	LC	LC	29e	LC	LC
18	LC	LC	29f	179.4	89.5
21	LC	LC	29g	239	183
24	LC	LC	29h	239	186.3
28a	42.6	50.4	29i	LC	LC
28b	30.5	18.9	29j	LC	LC
28c	HC[b]	HC	29k	LC	LC
28d	49.3	90.3	29l	LC	LC
28e	24.6	71.8	29m	LC	LC
28f	LC	175	30	LC	LC
28g	LC	225	31	LC	193
28h	LC	147	32	LC	LC

[a] LC: low cytotoxicity, CC_{50} > 250 μM.
[b] HC: high cytotoxicity, CC_{50} < 3.9 μM.

Analysis of the structure–activity relationships revealed that the alcohol derivatives **6** and **7** were less active than the lead compound Arbidol against all virus strains, indicating the importance of the amine at position 4 for Arbidol activity; while the presence (**7**) or absence (**6**) of bromine had little effect. The insertion of a methylene spacer between the indole ring and hydroxyl group caused a substantial increase in activity of compound **15** (relative to **6**), which proved to be the most potent compound of Series I (Table 1A). It was very potent against almost all the subtypes of influenza A tested, with IC_{50} values ranging from 3.9 to 31.3 μM, except for A/ty/Italy/2002(H7N3) and the influenza B virus. In particular, its inhibitory activity was better than Arbidol against four virus strains, A/California/7/2009(H1N1pdm), A/X31(H3N2), A/dk/Czechoslovakia/56(H4N6) and A/dk/Memphis/74(H11N9), and was comparable against A/Wuhan/359/95(H3N2). The greater potency of compound **15** is accentuated by its lower cytotoxicity than Arbidol; consequently the therapeutic indices are generally higher than those of the lead compound (Table 3A**)**.

The insertion of the methylene spacer in **7**, which retains the Br substituent, resulted in loss of activity of compound **21**. Compound **18**, in which the thiophenyl substituent at position 2 was replaced by a methyl group and bromine was retained in position 6, was even more potent than Arbidol against some of the viruses, while **16**, which lacks the Br moiety, was much less active (Tables 1A and 3A).

Compounds of the 'amino' Series II, in which the hydroxyl group at position 5 is replaced by an amine, that have a methyl group in position 2 (**24, 29a–b, 29d–m, 30, 31**) exhibited low activity or were completely inactive, except for compound **29c** that showed good potency (IC_{50} ranged from 7.9 μM to 31.3 μM) against all virus strains, but was very cytotoxic (Tables 1A and 2). Compounds **28a–e**, which retained the thiophenyl substituent, also exhibited greater potency than Arbidol, but were more cytotoxic.

For compounds with low cytotoxicity and high inhibitory activity, the therapeutic indices (TI; defined as the ratio between the CC_{50} and the IC_{50}) are shown in Tables 3A and 3B**.** Based on these data, compound **15** was selected for further experiments because it showed a better therapeutic index for most viruses tested, compared to Arbidol and the others analogues, except for **18** which

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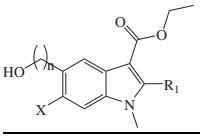
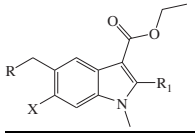
Table 3A
Therapeutic indices (TI) of Arbidol and Series I compounds in inhibition of the replication of influenza viruses.

Cpds				Virus tested TI									
	X	***n***	***R*** $_1$	H1N1[a]	H1N1[b]	H1N1pdm[c]	H3N2[d]	H3N2[e]	H4N6[f]	H7N3[g]	H8N4[h]	H11N9[i]	B[j]
6	H	0	CH_2SPh	8.0	4.0	8.0	8.0	8.0	4.0			4.0	
7	Br	0	CH_2SPh	4.0	1.0	8.0	2.0	1.0	4.0			1.0	
15	H	1	CH_2SPh	16.0	8.0	16.0	16.0	64.1	25.0	2.0	8.0	8.0	4.0
16	H	1	CH_3	–	–	–	–	–	2.0	–	–	2.0	–
18	Br	1	CH_3	31.6	2.0	31.6	–	–	31.6	8.0	31.6	31.6	–
21	Br	1	CH_2SPh					4.0	2.0		2.0	2.0	
ARB		10.0	7.5	5.0	7.5	15.0	3.7	7.5	7.5	–	3.7		

[a] A/Brisbane/59/07.
[b] A/PR/8/34.
[c] A/California/7/09.
[d] A/Wuhan/359/95.
[e] A/X31.
[f] A/dk/Czechoslovakia/56.
[g] A/ty/Italy/02.
[h] A/ty/Ontario/68.
[i] A/dk/Memphis/74.
[j] B/Brisbane/60/06.

Table 3B
Therapeutic indices of Arbidol and Series II compounds in inhibition of the replication of influenza viruses.

Cpds				Virus tested TI									
				H1N1[a]	H1N1[b]	H1N1pdm[c]	H3N2[d]	H3N2[e]	H4N6[f]	H7N3[g]	H8N4[h]	H11N9[i]	B[j]
28a	H	CH_2SPh	$N(CH_3)_2$	6.4	3.2	6.4	6.4	12.9	6.4	1.6	6.4	6.4	1.6
28b	H	CH_2SPh	$N(CH_2CH_3)_2$	7.8	2.0	7.8	3.9	7.8	3.9	1.0	3.9	3.9	1.0
28d	H	CH_2SPh	Pyrrolidine	11.4	2.9	11.4	11.4	5.8	11.4	1.9	5.8	11.4	0.7
28e	H	CH_2SPh	N-methyl piperazine	9.1	2.3	9.1	9.1	4.6	9.1	2.3	4.6	9.1	–
28f	H	CH_2SPh	NHBoc-piperazine	8.0		8.0		4.0					
28g	H	CH_2SPh	N-(2,4 difluoro)phenylpiperazine	8.0		8.0		4.0					
28h	H	CH_2SPh	Morpholine	8.0		8.0		4.0					
29c	H	CH_2SPh	$N(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$	6.6	6.6	6.6	3.3	6.6	3.3	3.3	1.7	3.3	1.7
29d	H	CH_3	Pyrrolidine	8.0	–	8.0	8.0	4.0	2.0	2.0	–	–	–
29e	H	CH_3	*N*-methyl-piperazine	8.0		8.0	8.0	4.0	2.0	2.0	–	–	–
29f	H	CH_3	2-piperazin-1-ylethylamino	5.7		5.7		2.9					
29g	H	CH_3	$N(CH_3)CH_2CH_2N(CH_2CH_3)_2$	7.6		7.6		–					
29h	H	CH_3	$N(CH_2CH_3)CH_2CH_2N(CH_2CH_3)_2$	7.6		7.6		–					
29i	H	CH_3	$NHCH_2CH_2NHCH_3$	8.0		8.0		4.0					
29j	H	CH_3	$NHCH_2CH_2NHBoc$	8.0		8.0		4.0					
29 k	H	CH_3	$NHCH_2CH_2N(CH_3)_2$	8.0		8.0		4.0					
29 l	H	CH_3	$NHCH_2CH_2OH$	8.0		8.0		4.0					
29 m	H	CH_3	$NHCH_2CH_2NHCH_2CH_2OH$	8.0		8.0		16.0					
30	H	CH_3	$N(CH_3)_2$	4.0	2.0	2.0	16.0	–	–	–	2.0	2.0	2.0
31	H	CH_3	Diazepan	–	2.0	–	–	–	–	2.0	–	–	–
32	H	CH_3	*N*-methyl-pyrrolidinium iodide	8.0		8.0		4.0					
ARB				10.0	7.5	5.0	7.5	15.0	3.7	7.5	7.5	–	3.7

[a] A/Brisbane/59/07.
[b] A/PR/8/34.
[c] A/California/7/09.
[d] A/Wuhan/359/95.
[e] A/X31.
[f] A/dk/Czechoslovakia/56.
[g] A/ty/Italy/02.
[h] A/ty/Ontario/68.
[i] A/dk/Memphis/74.
[j] B/Brisbane/60/06.

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Table 4
Influence of single amino acid substitutions in HA of X31 on the effects of **15** and Arbidol on the pH of fusion, determined by haemolysis assay, and on the IC_{50} for inhibition of virus replication.

Virus	pH of fusion	ΔpH of fusion		IC_{50}	
	Control	Arbidol	15	Arbidol	15
X31	5.2	−0.3	−0.4	7.8 (2.3)	3.9 (2.3)
1a (HA2 D112G)	6.0	−0.3	−0.5	62.5 (6.4)	62.5 (6.8)
2a (HA2 R54K)	5.7	−0.2	−0.2	62.5 (2.2)	62.5 (6.9)
ab4 (HA1 H17R)	6.1	−0.7	−0.7	62.5 (5.8)	125 (12.3)
V9A (HA1 G218R)	5.5	−0.4	−0.5	31.3 (9.1)	31.3 (9.1)

was very potent against several subtypes, but showed no activity against influenza A(H3N2) and B viruses.

3.2. Activities of *15* and Arbidol against the replication of X31 fusion mutants

To better understand the interaction of compound **15** with the virus HA, relative to that of Arbidol, we compared their activities against mutants of X31 with increased fusion pH. The results (Table 4) show that, as for Arbidol, inhibition of virus replication by **15** was substantially reduced, with IC_{50} 8 to 32-fold higher than for wild type X31. Thus the amino acid substitutions which reduce the acid stability of the HA had a similar effect on inhibition by **15** and Arbidol, consistent with a similar mechanism of action, and possibly interaction with a similar site on the protein.

3.3. Inhibition of HA-mediated membrane fusion

The influence of **15** and Arbidol on HA-mediated membrane fusion was studied using two assays, which monitor the pH-dependence of virus-induced haemolysis and of HA-mediated heterokaryon formation. The former assay allowed analysis of the effects of the amino acid substitutions in HA on the pH of fusion as well as on the influence of the inhibitors (Table 4).

For each virus, addition of 40 μM of **15** or Arbidol caused a decrease in the pH at which 50% haemolysis occurred, the decrease due to **15** being somewhat greater than that due to Arbidol for two of the four viruses, consistent with its greater potency against wild type virus replication. Thus the amino acid substitutions in the mutant HAs did not abolish the interaction of the inhibitor, but simply shifted the pH at which fusion occurred. Only in the case of mutant V9A was the pH of haemolysis reduced below that of wild type X31, which correlates with the lower IC_{50} against replication of this mutant. The marked difference in the magnitude of the effects on the mutants 2a and ab4, one lower and the other greater than on wild type virus, respectively, suggests differing effects of the mutations on inhibitor interaction, as well as HA stability.

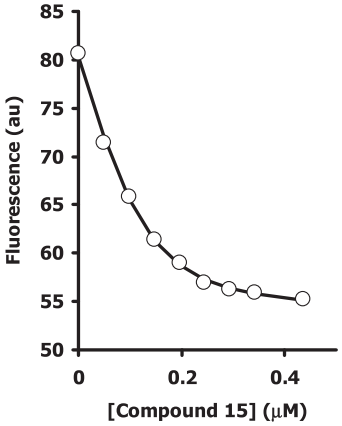

Fig. 2. Quenching of tryptophan fluorescence of X31 BHA by 15.

Equivalent results were obtained for the effects of the inhibitors on the pH-dependence of heterokaryon formation of CHO cells expressing the X31 HA, in the absence or presence of **15** or Arbidol (Fig. 1). At pH 5.2 there was little observable effect of either pH or inhibitor on the appearance or integrity of the cell monolayer. At pH 5.0 heterokaryon formation was evident only in the minus inhibitor control, while at pH 4.8 heterokaryon formation was evident in the presence of Arbidol (40 μM), but not in the presence of **15** (40 μM), indicating a greater effect of the latter in increasing acid stability. Thus **15**, like Arbidol, altered the pH of HA-mediated membrane fusion by increasing the acid stability of the HA.

3.4. Binding of *15* and Arbidol to HA

Two assays were used to monitor the interaction of **15** and Arbidol with purified HA: quenching of tryptophan fluorescence and fluorescence-based thermal shift assays. Fig. 2 shows the decrease

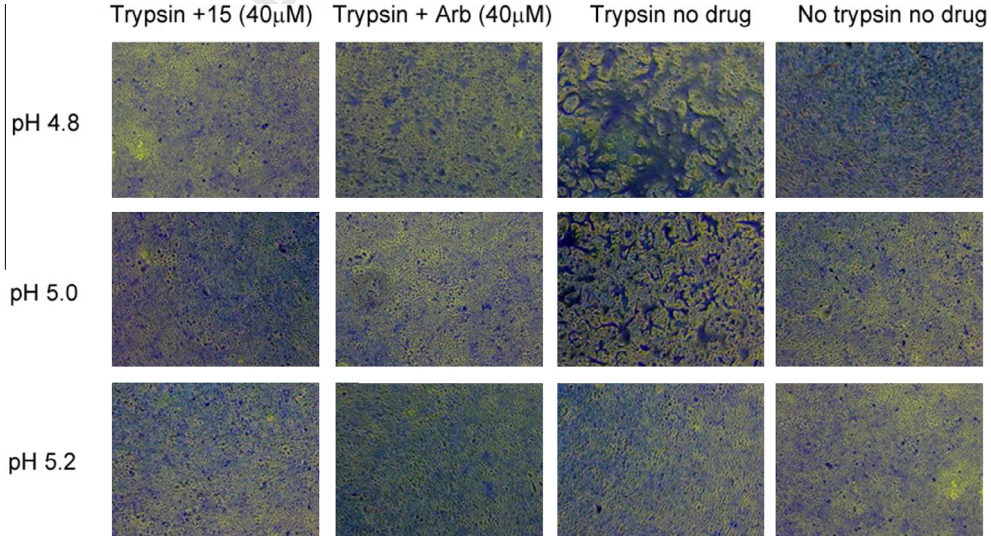

Fig. 1. Effects of inhibitors on the pH-dependence of heterokaryon formation of CHO cells expressing X31 HA, in the presence and absence of 15 or Arbidol.

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Table 5
Binding of Arbidol and **15** to purified BHAs of different subtypes, determined by quenching of tryptophan fluorescence. Results are expressed as Kd in μM (+/– standard deviation).

Virus	HA subtype	Arbidol	**15**
X31	H3	5.6 (0.7)	0.032 (0.006)
A/dk/Czechoslovakia/56	H4	7.9 (1.5)	0.078 (0.013)
PR8	H1	41.9 (5.2)	13.6 (2.1)
A/Brisbane/59/2007	H1	18.8 (2.9)	7.0 (1.2)
A/Singapore/1/57	H2	44.3 (7.1)	24.8 (5.2)
RG14 (A/Vietnam/1194/2004)	H5	28.3 (6.1)	24.5 (8.6)

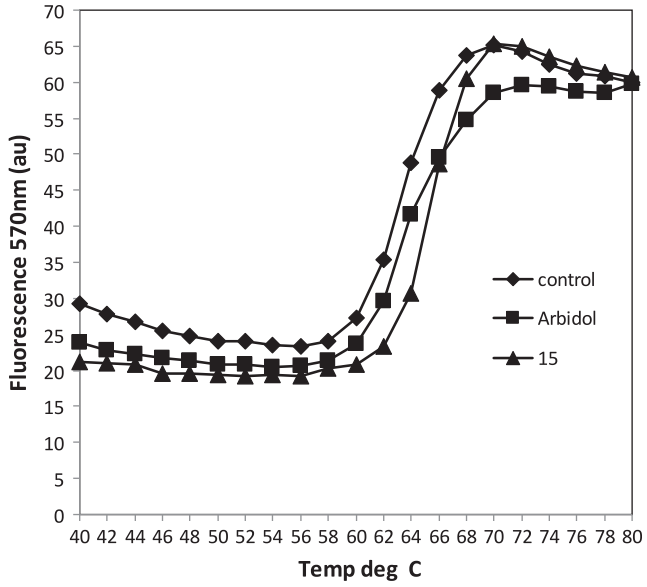

Fig. 3. Thermal shift of X31 BHA in the presence and absence of 15 or Arbidol.

in Trp fluorescence (at 340 nm) of X31 HA with increasing concentration of compound **15**. The maximum decrease in fluorescence was similar for the different HAs tested, within the range 32–43% for **15** and 36–52% for Arbidol. Table 5 compares the affinities of binding of **15** with those determined for Arbidol (Liu et al., in preparation) to HAs of different subtypes. While the affinities of binding of **15** to group 1 HAs, H1, H2 and H5, were up to 3-fold greater than those for Arbidol, the affinities for group 2 HAs were 100- and 175-fold greater, for H3 and H4, respectively. Thus **15** had a much greater preference (up to 750-fold) for binding to group 2 HAs than to group 1 HAs; differential binding by Arbidol was only up to 8-fold.

The thermal shift assay, which measures the influence of ligands on the thermal stability of the protein, also showed that **15** exerted greater stabilization of X31 HA (Fig. 3), and had greater affinity of binding, than Arbidol; T_ms were 66 °C and 64.5 °C, respectively, compared with 63 °C for wild type HA. The shift in T_m of 3 °C by **15** was similar to that caused by TBHQ (Russell et al., 2008). Similar T_m values were obtained by monitoring denaturation by Trp fluorescence, but interpretation of the data was complicated by the quenching effects of the ligands.

4. Discussion

On the one hand, while many of the derivatives of Arbidol exhibited reduced inhibitory activity, significant changes to the structure of Arbidol could be made while retaining inhibitory activity against virus replication. For example, the removal of the amine at position 4 and Br at position 6 was complemented by insertion of a methylene extension of the hydroxy group at position 5 of **15**, while retention of the Br substituent was necessary to complement the removal of the thiophenyl group from position 2 in **18**. Although compounds in which the hydroxyl of **15** was replaced by an amino substituent retained inhibitory activity, they were in general more cytotoxic.

On the other hand, it is apparent that the degree of inhibition of the replication of different viruses, in terms of IC_{50}, do not directly reflect differential interaction with HA or its consequences. Thus although **15** or Arbidol increase the acid stability of mutant HAs with elevated pH of fusion, e.g. mutant 1a (fusion pH 6.0) by a similar degree to that of wild type HA (fusion pH 5.2), they cause poor inhibition of virus replication. This is readily understood in terms of the pH of fusion of the HA in relation to the pH of the endosome which triggers the conformational change in HA to promote fusion. The conformational change in the stabilized, inhibitor bound, mutant HA (for 1a, 2a and ab4) occurs at a pH higher than that of the wild type HA and is thus still triggered by the endosomal pH and hence refractory to the stabilizing effects of the ligands. Such an effect may account for the somewhat greater sensitivity of the replication of X31 (fusion pH 5.2) than of A/dk/Czechoslovakia/56 (fusion pH 6.1), the HAs of which bind **15** and Arbidol with similar affinities; however, the inhibition of A/dk/Czechoslovakia/56 replication is not as severely impaired by the high fusion pH as for mutant ab4. Differences in the effects of inhibitor binding to mutant HAs 1a and ab4 (with similar fusion pH to A/dk/Czechoslovakia/56) on the pH of fusion also indicate that other features of the HA influence the consequences of ligand binding, both in terms of pH of fusion and inhibition of virus replication. In the absence of a difference in the pH of fusion of PR8 and X31 HAs, both estimated to be about 5.5 from a combination of comparative haemolysis and other assays (S. Wharton, unpublished), the somewhat greater sensitivity of X31 replication to **15** may reflect to a small extent the 430-fold greater affinity of **15** for the X31 HA than PR8 HA. It is evident, however, that differences in affinity of inhibitor binding are not reflected in the relative degree of inhibition of virus replication and that inhibition of different A subtype viruses by **15** did not show any clear segregation between those with group 1 or group 2 HAs, although **15** was somewhat more active against the H3 and H4 (group 2), than against H1, H8 and H11 (group 1) viruses. The much greater binding affinity of **15** than Arbidol to the group 2 HAs at pH 6.5 was not reflected in a marked increase in potency of **15** compared to Arbidol against group 2 virus replication.

An explanation for the greater inhibition of the replication of group 2 than group 1 viruses by TBHQ was provided by crystallographic data of a X31 HA-TBHQ complex which indicated less space to accommodate the inhibitor in the H1 HA structure compared with H3 HA (Russell et al., 2008). In the absence of equivalent structural information on the interaction of compound **15** and Arbidol with HA we have no explanation for the different relative binding of **15** and Arbidol to Group 2 and group 1 HAs, or for the differences observed in inhibition of virus replication, which occurred at a similar concentration (5–10 μM) (Bodian et al., 1993) for TBHQ as for **15** and Arbidol.

Since the fluorescence intensity of the conserved Trp92 of HA2 (solvent inaccessible) was shown to be substantially greater than that of other Trp residues in HA (Wharton et al., 1988), it is likely that it is the fluorescence of this residue which is quenched by Arbidol and **15**. Furthermore, the proximity of Trp92 to the TBHQ binding site (Russell et al., 2008) suggests that **15** and Arbidol may bind to a similar site. Due to the intrinsic fluorescence of TBHQ it was not possible, however, to use

this assay to compare the relative binding affinities of **15** and TBHQ to the different HAs.

Arginine 54, substituted in HA2 of mutant 2a, contributes to the TBHQ binding site. The reduced stabilising effect of **15** on the R54K HA (0.2 pH units) compared to the other mutants or wild type HAs (0.4–0.7 pH units) (Table 4) is consistent with a direct effect of the mutation on the binding of **15** to a site close to that bound by TBHQ. In contrast substitution in the other residues, in particular H17R in HA1, located close to the fusion peptide and involved in stabilising its location, tend to accentuate the effect of **15**, rather than impede its interaction.

In view of its greater sensitivity to differences in structure of inhibitor and HA, the direct binding assay should prove more illuminating in future SAR studies. Further information including structural data on ligand-HA complexes will be required to understand the bases for the differential specificities of binding of arbidol and its derivatives and differences in the spectrum of inhibition of influenza virus replication by different classes of fusion inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.05.005.

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