Narcissus tazetta lectin shows strong inhibitory effects against respiratory syncytial virus, influenza A (H1N1, H3N2, H5N1) and B viruses

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A mannose-binding lectin (*Narcissus tazetta* lectin [NTL]) with potent antiviral activity was isolated and purified from the bulbs of the Chinese daffodil *Narcissus tazetta* var. *chinensis*, using ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose, affinity chromatography on mannose–agarose and fast protein liquid chromatography (FPLC)-gel filtration on Superose 12. The purified lectin was shown to have an apparent molecular mass of 26 kDa by gel filtration and 13 kDa by SDS–PAGE, indicating that it is probably a dimer with two identical subunits. The cDNA-derived amino acid sequence of NTL as determined by molecular cloning also reveals that NTL protein contains a mature polypeptide consisting of 105 amino acids and a C-terminal peptide extension. Three-dimensional modelling study demonstrated that the NTL primary polypeptide contains three subdomains, each with a conserved mannose-binding site. It shows a high homology of about 60%–80% similarity with the existing monocot mannose-binding lectins. NTL could significantly inhibit plaque formation by the human respiratory syncytial virus (RSV) with an IC_{50} of 2.30 μg/ml and exhibit strong antiviral properties against influenza A (H1N1, H3N2, H5N1) and influenza B viruses with IC_{50} values ranging from 0.20 μg/ml to 1.33 μg/ml in a dose-dependent manner. It is worth noting that the modes of antiviral action of NTL against RSV and influenza A virus are significantly different. NTL is effective in the inhibition of RSV during the whole viral infection cycle, but the antiviral activity of NTL is mainly expressed at the early stage of the viral cycle of influenza A (H1N1) virus. NTL with a high selective index ($SI=CC_{50}/IC_{50} \geq 141$) resulting from its potent antiviral activity and low cytotoxicity demonstrates a potential for biotechnological development as an antiviral agent.

1. Introduction

Many plant lectins are known to possess antiviral properties against human enveloped viruses (Balzarini 2007a). They play important biological roles both within and outside the plants. A majority of plant lectins are involved in defence. In many plants, lectins are usually associated with storage tissues such as seeds, tubers, bulbs, corms, rhizomes, root

stocks and bark (Van Damme *et al.* 1995). The antiviral activities of various lectins have been demonstrated against the human immunodeficiency virus (HIV) (Balzarini *et al.* 1992; Balzarini 2006), human cytomegalovirus (HCMV) (Balzarini *et al.* 1991, 1992), hepatitis C virus (HCV) (Bertaux *et al.* 2007), herpes simplex virus type 1 (HSV-I) (Ooi *et al.* 2004a), herpes simplex virus type 2 (HSV-2) (Luo *et al.* 2007), influenza A (H1N1) virus (Ooi *et al.* 2004b), poxvirus (Kaur *et al.* 2007), respiratory syncytial virus (RSV) (Ooi *et al.* 2004b) and coronavirus (SARS-CoV) (Keyaerts *et al.* 2007). There has been increasing interest in recent years in the special characteristics of lectins derived from monocot plants (Van Damme *et al.* 1995). The monocot mannose-binding lectins belong to a superfamily of structurally and evolutionarily related proteins (Barre *et al.* 1996). This group of lectins shares a number of biological properties, such as rabbit erythrocyte haemagglutination, antiviral activity (Balzarini *et al.* 2006, 2007b; Keyaerts *et al.* 2007) and insecticidal effect (Gatehouse *et al.* 1995; Hogervorst *et al.* 2006; Ohizumi *et al.* 2009).

Mannose-binding lectins with antiviral activity have also been isolated from the leaves and bulbs of *Narcissus tazetta* var. *chinensis*, commonly known as the Chinese daffodil, which is a monocot perennial ornamental plant of the family Amaryllidaceae (Ooi *et al.* 1998, 2000). A potent antiviral lectin from *N. tazetta* (designated NTL) exists as a homodimer consisting of two identical subunits of 13 kDa (Ooi *et al.* 2000, 2001). In mouse, intraperitoneal injection of NTL modulates the gene expression of immunoregulatory cytokines such as interferon (IFN)-*γ*, transforming growth factor (TGF)-*β* and stem cell factor (SCF) in macrophages and/or spleen cells (Ooi *et al.* 2002). Furthermore, NTL is shown to preserve haematopoietic stem and progenitor cells after 35 days of culture in a serum- and cytokine-free condition, and enhance *ex vivo* expansion of severe combined immunodeficiency (SCID)-repopulating cells (Li *et al.* 2008). The dual functions of NTL in preservation of early stem and progenitor cells, and expansion of lineage-committed cells in prolonged culture could be developed for clinical expansion of CD34+ cells for transplantation and use of these cells for other cell therapy strategies. In this study, we investigated the inhibitory effect of a novel mannose-binding lectin isolated, purified and cloned from the bulbs of the Chinese daffodil, *Narcissus tazetta* var. *chinensis,* against human RSV, and various strains of influenza A (H1N1, H3N2, H5N1) and influenza B viruses.

2. Materials and methods

2.1 *Isolation and purification of* Narcissus tazetta *lectin (NTL)*

Lectins from the bulbs of the Chinese daffodil, *Narcissus tazetta* var. *chinensis,* were isolated and purified as described

previously (Ooi *et al.* 1998, 2000), using ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose, affinity column chromatography on mannose–agarose and fast protein liquid chromatography (FPLC)-gel filtration on a Superose 12 column. Haemagglutinating activity was determined using serially two-fold dilutions of the lectin solution in microtitre U-plates (50 μl) mixed with 50 μl of a 2% suspension of rabbit erythrocytes in pH 7.2 phosphate-buffered saline (PBS) at room temperature. Specific activity was regarded as the number of haemagglutination units per mg protein.

The crude proteins obtained by extraction with aqueous buffer, ammonium sulphate precipitation and DEAE-cellulose column chromatography were dissolved in 20 mM MES buffer of pH 6.2 and chromatographed on a mannose–agarose (Sigma) column. Unadsorbed proteins (the M1 fraction) were eluted with MES buffer, and adsorbed proteins were desorbed with MES buffer containing 0.2 M mannose to give the M2 fraction. The M3 fraction was obtained after washing the column with MES buffer containing 0.5 M NaCl. The fractions were desalted and mannose was removed by dialysis and the proteins were lyophilized. Proteins with different molecular masses in the M2 and M3 fractions were further separated by gel filtration on a Superose 12 column using the FPLC system (Pharmacia), which gave rise to the relatively purified fractions NTM2 and NTM3, respectively. The protein profiles of various fractions were studied and their molecular masses determined by sodium dodecyl sulphate (SDS)-PAGE. Briefly, proteins were mixed with loading dye (1:1) with 2% SDS and 2% *β*-mercaptoethanol and denatured for 5 min at 95ºC. A low range SigmaMarker™ (Sigma) and the proteins were then resolved using 15% SDS-polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 for 1 h and subsequently destained with 10% acetic acid and 40% methanol. Pure NTL (from the NTM2 fraction) was obtained after desalting the proteins with a PD-10 column (Bio-Rad, Hercules, CA, USA) and analysed by SDS-PAGE (figure 1). The N-terminal amino acid sequence was also determined using a Hewlett Packard (HP) G1000A Edman degradation unit and an HP 1000 HPLC system.

2.2 *Modelling of three-dimensional (3D) structure of NTL*

The cDNA-derived amino acid sequence of NTL was determined by molecular cloning as described previously (Ooi *et al.* 2001). Its primary protein structure contains a mature polypeptide consisting of 105 amino acids and a C-terminal peptide extension beyond the C-terminal amino acids Thr–Gly (table 1). The homology model was built on the basis of amino acid sequence of the well-known lectin GNA (mannose-specific agglutinin from the bulbs of the snowdrop *Galanthus nivalis*) as the template (Gene Bank
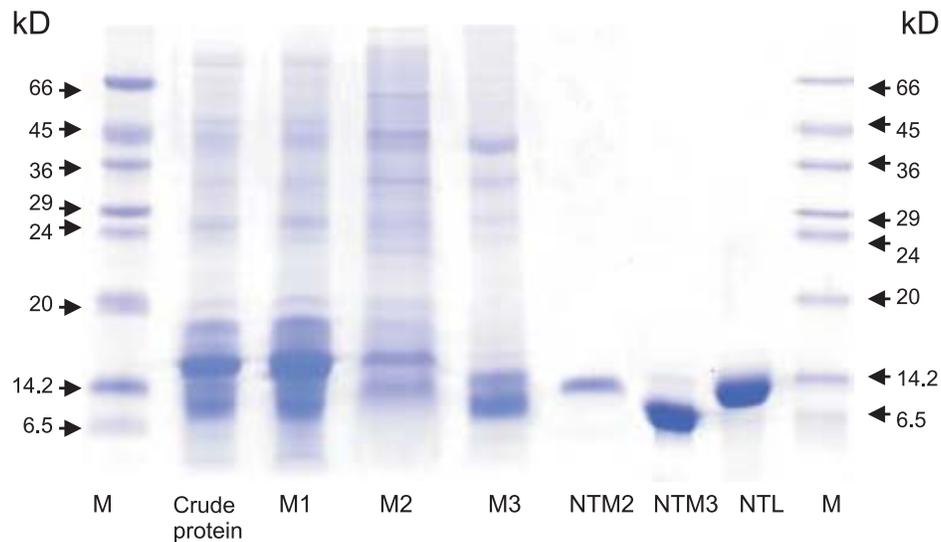
Figure 1. SDS-PAGE of *Narcissus tazetta* lectin (NTL) and various fractions of proteins during the purification process. Proteins were separated by electrophoresis using 15% SDS-polyacrylamide gels and stained by Coomassie brilliant blue R-250. M, low range SigmaMarker™

Table 1. Plaque formation assay for respiratory syncitial virus (RSV) in Hep2 cells – IC_{50}, CC_{50} and SI values of NTL

IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
2.30	325.40	141.36

IC_{50} – concentration that leads to 50% of maximal inhibition on the number of plaques formed by RSV as compared with the virus control

CC_{50} – 50% cytotoxic concentration (CC_{50}), which is the concentration of substances that inhibit up to 50% of the viability of cells

SI – selectivity index (CC_{50}/IC_{50})

CC_{50} and IC_{50} were calculated from data obtained from three independent experiments with the generation of dose the response curves by GraphPad Prism 4.0 software.

Database No. AAA33346.1). Its percentage of sequence identity with NTL is 67.619%. Three-dimensional (3D) structure predictions of the NTL polypeptide were conducted by using the SWISS-MODEL program (ExPASy proteomic tools, available from http://ca.expasy.org/tools/) and drawn by the RasMol software (figure 2).

2.3 *Cells and viruses for experimental use*

Human larynx epidermoid carcinoma cell line (HEp-2) cells, Madin–Darby canine kidney cell line (MDCK) cells and RSV (Long strain) were purchased from the American Type Culture Collection (ATCC). All influenza A (three strains, i.e. H1N1, H3N2 and H5N1) and influenza B viruses were clinical isolates obtained from the Department
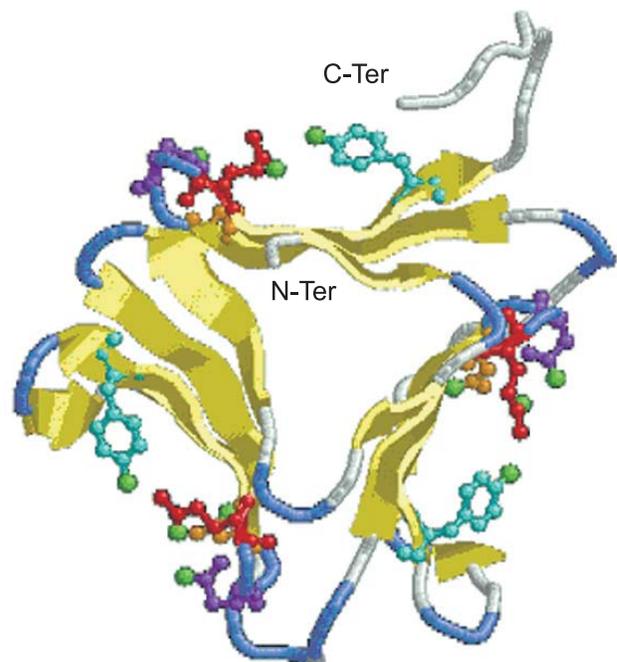
Figure 2. Cartoon diagram of *Narcissus tazetta* lectin (NTL) protein structure simulated using the Swiss-Model program and RasMol software. Three bundles of β-sheets oriented perpendicular to the axis of a prism are shown in yellow. The amino acid residues Q_D_N_Y constituting the three mannose-binding sites are highlighted with ball–stick spatial configurations (Q, red; D, purple; N, orange; Y, cyan). Green balls indicate oxygen atoms predicted to form hydrogen bonds with O2, O3, O4 of mannose. C-Ter, C-terminal; N-Ter, N-terminal

of Microbiology, Prince of Wales Hospital, Hong Kong SAR, China. The cells were cultured in the growth medium comprising Eagle minimum essential medium (EMEM) (Invitrogen, California, USA), and were incubated at 37°C in a humidified 5% CO_2 atmosphere. All media were supplemented with 10% foetal bovine serum (FBS) (Invitrogen, California, USA), except in assays for influenza viruses. Infections with human influenza viruses, namely, A(H1N1)/HongKong/CUHK-13003/2002, A(H3N2)/Hong Kong/CUHK-22910/2004, A(H5N1)/HongKong/483/1997, and B/HongKong/CUHK-24964/2004 (Influenza B) were carried out in serum-free medium formulated with 1 μg/ml of trypsin treated with tolylsulphonyl phenylalanyl chloromethyl ketone (TPCK-treated trypsin) (Sigma-Aldrich, Munich, Germany).

For RSV culture, 1.75×10^4 HEp-2 cells in 100 μl growth medium were seeded into each well of a 96-well tissue culture plate and incubated for 24 h. After removing the growth medium, the RSV stock was diluted using EMEM supplemented with 1% FBS (maintenance medium) and added to the subconfluent HEp-2 cells (100 μl/well). The plate was observed daily under the microscope for any cytopathic effects (CPE) induced by the virus. When the maximal formation of syncytia was observed, the supernatant was collected and centrifuged at 4°C, 2000x*g* for 10 min to remove cell debris. Aliquots of the supernatant were frozen at –80°C until use.

2.4 *Cytotoxicity assays for MDCK cells and HEp-2 cells*

The cytotoxicity of NTL on MDCK cells was measured quantitatively by the reduction of formazan dye using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann 1983). MDCK cells were seeded at 80% confluence in a 96-well microtitre plate (Nunc,
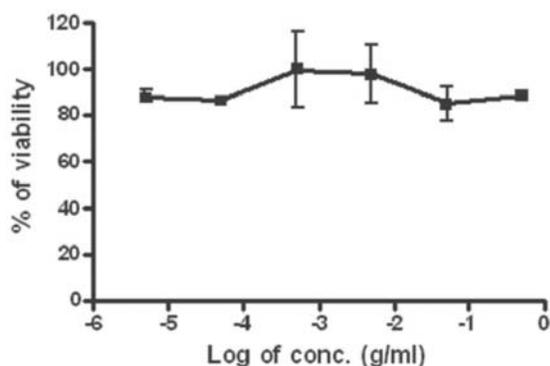

Figure 3. The cytotoxic effect of *Narcissus tazetta* lectin (NTL) (dosages ranging from 0.05 μg/ml to 500 μg/ml) in MDCK cells as determined by MTT assay after 24 h incubation. Data are the mean ± SD of two independent experiments.

Denmark) with 10-fold dilutions of NTL in culture medium. Morphological changes on the MDCK cells were observed microscopically after 24 h of incubation (figure 3).

For studying the cytotoxicity of NTL on HEp-2 cells using MTT assay, 2-fold serially diluted NTL samples were added to cells at 25% confluence and incubated for 48 h. After removal of the medium, 50 μl of MTT (0.5 mg/ml in PBS) was added into each well and incubated for 3 h at 37°C. Dimethyl sulphoxide (DMSO) (100 μl/well) was then added to dissolve the water-soluble formazan. The optical density (OD) was quantified by a micro-spectrophotometer at 540/630 nm. The reduction of formazan at each concentration was determined as the percentage of cell control. Cytotoxicity is expressed as 50% cytotoxic concentration (CC_{50}), which is the concentration of the substance that inhibits up to 50% of viable cells (table 2). The percentage of cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = (\text{Exp. value} - \text{low control}) / (\text{High control} - \text{low control}) \times 100.$$

2.5 *Antiviral assays*

2.5.1 *Anti-influenza assay using extracellular virus yield reduction method:* Extracellular virus yield reduction assay was performed by growing the influenza viruses in the presence of NTL as described by Smee *et al.* (2001). The MDCK cells were incubated with serially 2-fold dilutions of NTL for 30 min in a 24-well plate (Nunc, Denmark), and then challenged with the influenza viruses at a multiplicity of infection (MOI) of approximately 0.001 PFU/cell for 24 h at 37°C. The virus-containing supernatants (extracellular virus), after one freeze–thaw cycle, were further titrated by plaque assay adopted from Tobita *et al.* (1975). Non-infected cell controls and virus controls were performed in each experiment. Data were reported as the effective concentration of the compound required to inhibit viral infectivity by 50% (IC_{50}) (figure 4).

2.5.2 *Mode of anti-influenza action – haemagglutination (HA) assay:* To study the mode of action of NTL against influenza viruses, the virus titres from cultures treated with the drug 1 h prior to or post-virus inoculation were compared. Confluent MDCK cells in 24-well plates were incubated with or without NTL (15 μg/ml) for 1 h at 37°C. The cells were then rinsed with PBS and challenged with 10-fold serial dilutions of viruses in the presence or absence of NTL. After 1 h of viral adsorption at 37°C, cells were rinsed with PBS and further incubated with NTL for 72 h. The virus-containing supernatants, after one freeze–thaw cycle, were subsequently titrated by haemagglutination (HA) assay. Cell controls and virus controls were performed in parallel in each experiment. A significant virus-inhibitory effect was indicated by a reduction of HA titre by 2$\log_2$ or more (Serkedjieva and Velcheva 2003).
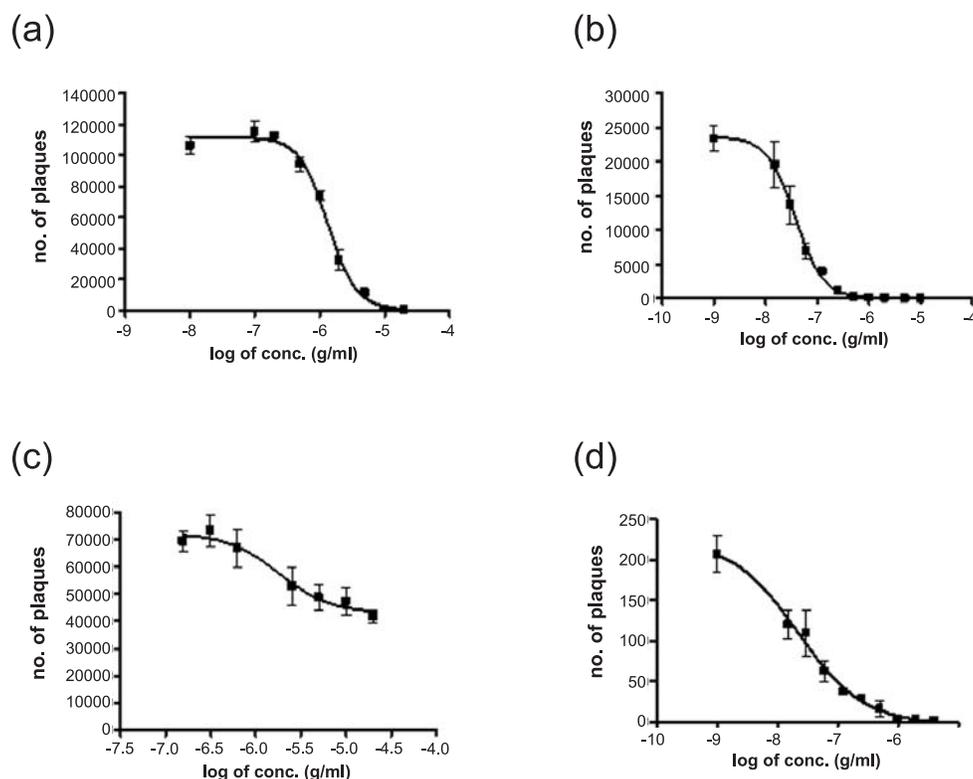
Figure 4. The viral inhibition of *Narcissus tazetta* lectin (NTL) (0.001 µg/ml to 20 µg/ml) in MDCK cells infected with **(a)** H1N1, **(b)** H3N2, **(c)** H5N1, **(d)** influenza B viruses as determined by virus yield reduction assay. All data were derived from triplicate determinations.

Table 2. Deduced amino acid sequence of full-length cDNA encoding *Narcissus tazetta* lectin (NTL)

[1] DNILYMGETLYAGQFLNYGPYKFIIQDDCNLVLYTKSDPLWASNTGGLAT[50]

[51] GCRVTMQADGNLVVYSESNRAIWASNTNIGNGNYVLLLQKDRNVVIYGSS[100]

↓

[101]LWATGTYIGLLGIPGVAQNSTSVTSGKKWNNCKCVIFCMHNVPLAAWWES[150]

[151]FFMYCMGMLFSFK[163]

The full-length amino acid sequence of NTL contains a mature polypeptide domain consisting of 105 amino acids and a
C-terminal peptide extension beyond Thr–Gly (as indicated by an arrow).

2.5.3 *Plaque reduction assay for measuring inhibition by RSV:* For anti-RSV activity determination, HEp-2 cells were seeded in 12-well plates (3×10^5 cells/well) and incubated for 24 h. After removing the growth medium, RSV at about 80 PFU and 2-fold diluted NTL (both in the maintenance medium) were added to each well. For the virus control wells, only RSV was added. The plates were further incubated for 2 h with intermittent shaking. The cells were subsequently rinsed twice with PBS and overlaid with 1% agarose in the maintenance medium. After the agarose had solidified, an equal volume of sample or maintenance medium was added to the corresponding wells and virus control wells, respectively. The plates were then incubated for 3 days to allow the formation of plaques. Cells were

fixed and stained, and the number of plaques was counted under a light microscope. The percentage of inhibition by the samples on plaque formation was calculated and the 50% maximal inhibitory concentration on RSV-induced plaque formation by the sample was defined as the 50% inhibitory concentration (IC_{50}).

2.5.4 *Mode of anti-RSV action – time of addition study:* To study the mode of anti-RSV action, NTL and RSV were added to HEp-2 cells at different time points, so that NTL was present under different conditions: (1) during the early infection phase (the first 2 h post-infection) only; (2) during the whole infection cycle (sample present throughout the experiment), or (3) during the later infection phase only (sample only present after 2 h post-infection). HEp-2 cells at

3×10^5 cells/well were seeded in 12-well plates and incubated for 24 h. After removing the growth medium, 100 μl of RSV at about 80 PFU and 100 μl of NTL were added to each well with a final concentration of 10 μg/ml. For virus control wells and the wells of condition 3, 200 μl RSV was added at a final concentration of 80 PFU. After further incubation for 2 h, unbound RSV and samples were washed away by rinsing the cells twice with PBS. The cells were overlaid with 1% agarose in the maintenance medium (1 ml/well). After the agarose had solidified, 1 ml of the sample at double concentration (20 μg/ml) was added to each well of conditions 2 and 3. For each of the virus control wells and the wells of condition 1, 1 ml of maintenance medium was added. After plaque development, the number of plaques was counted and the percentage under different conditions as compared with the virus control was calculated. The results were analysed with one-way ANOVA followed by the Tukey multiple comparison test by the GraphPad Prism 4.0 software.

3. Results

NTL was isolated and purified from the bulbs of *N. tazetta* var. *chinensis* as described previously (Ooi *et al.* 1998, 2000). Purified NTL (derived from the NTM2 fraction) was obtained after FPLC-gel filtration followed by desalting with a PD-10 column, and its purity was analysed by SDS-PAGE (figure 1). It was determined to have a molecular mass of about 26 kDa by gel filtration and 13 kDa by SDS–PAGE. NTL is suggested to be a mannose-binding homodimer with two identical subunits of about 13 kDa. Molecular cloning revealed that the deduced amino acid sequence of the full-length cDNA encoding NTL contained a mature polypeptide consisting of 105 amino acids and a C-terminal peptide extension beyond the C-terminal amino acids Thr–Gly (table 1). There are two fixed-position cysteines within the protein domain (amino acids 29 and 52), which are probably involved in the disulphide-bond linkage within the molecules to confer the secondary structure of the mature lectin. One third of the deduced amino acid composition consisted of glycine, leucine and asparagines (table 1).

According to an NCBI conserved domain search, NTL protein possesses a *β*-lectin conserved domain, and 3 typical mannose-binding sites (Q_D_N_V_Y, from Q26 to Y34, Q57 to Y65 and Q89 to Y97, respectively) resembling many other monocot mannose-binding lectins, such as *Galanthus nivalis* (Van Damme *et al.* 1991; Hester *et al.* 1995; Barre *et al.* 2001). Four conserved amino acid residues (Gln, Asp, Asn and Tyr) in the mannose-binding sites were predicted to form hydrogen bonds with O2, O3 and O4 of mannose, and hydrophobic residue Val to interact with C3 and C4 of mannose through hydrophobic interactions. The 3D structure prediction of the NTL protein using SWISS-MODEL indicates that NTL is composed of 28 alpha helixes, 52 extended strands and 83

random coils, and that its monomer contains three subdomains, each with a conserved mannose-binding site (figure 2). Two subdomains were orthogonal with four-stranded beta-sheets and the other was orthogonal with three-stranded beta-sheets. The three subdomain bundles were asymmetrically arranged as a *β*-prism, and extended strands in each subdomain were perpendicular to the axis of the prism.

The results of the MTT cytotoxicity assay showed no significant reduction in viability of the MDCK cells (for culturing influenza) after 24 h incubation with NTL (figure 3), and no microscopic changes in cell morphology were observed. NTL also displayed low cytotoxicity (CC_{50}=325.4 μg/ml) as determined by MTT assay towards HEp-2 cells (for culturing RSV) (table 2).

The inhibitory effect of NTL on the replication of various strains of influenza viruses was evaluated by the extracellular virus yield reduction assay. NTL could potently inhibit the replication of various influenza subtypes in a dose-dependent manner (figure 4), and the anti-influenza activities were prominent against the H1N1, H3N2 and influenza B viruses with IC_{50} values of 1.33 μg/ml, 0.40 μg/ml, and 0.20 μg/ml, respectively. However, NTL exhibited only a moderate antiviral effect on the avian influenza A (H5N1) virus, with an IC_{50} higher than 20 μg/ml.

The time of addition study for influenza A (H1N1) virus using HA assay showed that there was a reduction of 5 $\log_2$ HA titre as compared with the controls when NTL was present throughout the entire virus adsorption and incubation period; whereas the reduction was 2 $\log_2$ HA titre when NTL was added only at 1 h following virus adsorption.
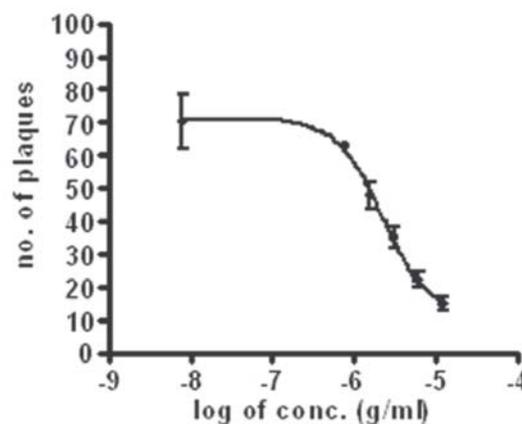

Figure 5. Inhibition of respiratory syncytial virus (RSV)-induced plaque formation by *Narcissus tazetta* lectin (NTL). The number of RSV plaques formed after the addition of different concentrations of NTL to infected HEp-2 cells was counted and a dose–response curve was generated by the GraphPad Prism 4.0 software. Data were obtained from three independent experiments and expressed as mean ± SD.
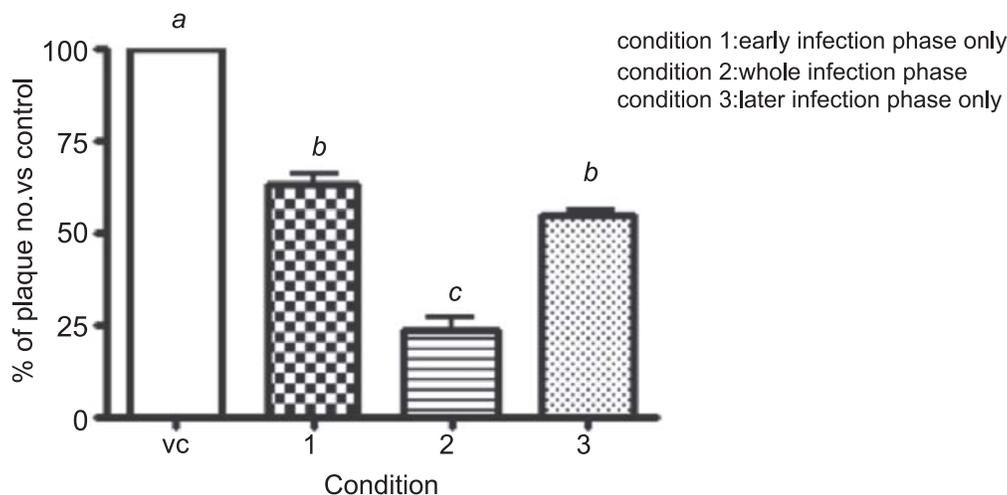
Figure 6. Mode of anti-respiratory syncytial virus (RSV) action of *Narcissus tazetta* lectin (NTL) by plaque formation assay. HEp-2 cells were infected with RSV at about 80 PFU and 10 μg/ml of NTL was added during the early infection phase only, during the whole infection cycle, or during the late infection phase only. The percentage of the number of plaques formed under different conditions as compared with the virus control (VC) was calculated and analysed by one-way ANOVA followed by Tukey multiple comparison post-test, *P*<0.001. Bars marked with the same letter have no significant difference. Results are expressed as mean ± SD obtained from three independent experiments with duplicate wells for each condition.

In an assessment for its anti-RSV activity using the plaque assay, NTL effectively inhibited plaque formation by RSV (IC$_{50}$=2.30 μg/ml) in a dose-dependent manner (figure 5), and had a selective index value (SI=CC$_{50}$/IC$_{50}$) of 141.36 (table 2). The result of a mechanistic study on anti-RSV action showed a significant reduction in plaque formation by RSV. The inhibition rate was 76.4% when NTL was present during the whole infection cycle of the virus, but was 36.6% when NTL was present only during the first 2 h of infection phase, and 45.7% if it was added only 2 h post-infection (figure 6).

4. Discussion

Mannose-binding lectins with antiviral activity have been isolated from the leaves and bulbs of some monocot plants (Balzarini 2007a, b; Balzarini *et al.* 1991, 1992; Ooi *et al.* 1998, 2004a, b; Keyaerts *et al.* 2007). Among these, a novel mannose-binding lectin isolated from *Narcissus tazetta* var. *chinensis*, known as NTL, is a homodimer with two subunits of about 13 kDa (Ooi *et al.* 2000, 2001). Molecular modelling displays that the three-dimensional structure of NTL polypeptide contains three subdomains, each with a conserved mannose-binding site. Protein–protein BLAST (UCBI) of the deduced amino acid sequence of NTL shows high similarities with existing monocot mannose-binding lectins, for example, *Allium ursinum* (Smeets *et al.* 1997)*, Amaryllis vittata* (Wu *et al.* 2004), *Dioscorea batatas* (Ohizumi *et al.* 2009), *Zephyranthes candida* (Wu *et al.* 2006) and *Galanthus nivalis* (Van Damme *et al.* 1991; Hester *et al.* 1995).

NTL possessed potent antiviral activities against RSV and various strains of influenza viruses. NTL could effectively inhibit RSV-induced plaque formation (IC$_{50}$= 2.30 μg/ml). Its cytotoxicity against HEp-2 cells was low (CC$_{50}$= 325.4 μg/ml) and thus it had a high SI value of 141.36. Similarly, the inhibition of replication of various influenza subtypes was clearly dose dependent. Viral infectivity with human influenza A (H1N1, H3N2) and influenza B viruses was strongly inhibited by NTL with EC$_{50}$ values ranging from 0.02 μg/ml to 1.33 μg/ml. However, NTL had weaker antiviral activity against the avian influenza (H5N1) virus than human influenza viruses, as no IC$_{50}$ for H5N1 virus can be determined only up to a concentration of 20 μg/ml. In parallel with antiviral activity, the cytotoxicity of NTL on MDCK cells was monitored by a colorimetric MTT assay. In concentrations of up to 500 μg/ml, no cell toxicity was observed by determining the effect on cell viability in MTT assay and cell morphology. The low cytotoxicity together with the high SI indicate that NTL possesses potential for biotechnological development as an antiviral agent against RSV and human influenza viruses, although potential application against avian influenza viruses remains to be determined.

Further investigation on its mode of action against RSV revealed that significant reduction in the number of plaques was observed when NTL was present during the whole viral infection cycle (inhibition by 76.43%). The inhibition rate was 36.57% if NTL was present only during the early infection phase (i.e. the first 2 h post-infection) and 45.73% if it was added in the later infection phase (i.e. sample only present 2 h post-infection). However, a similar investigation

on the antiviral activity of NTL against the H1N1 virus (A/HongKong/CUHK-13003/2002) indicated that the target of NTL was mainly in the early phase (i.e. virus adsorption phase). As the early infection events of influenza viruses were inhibited, NTL might be virucidal or, like some other antiviral lectins, it might interact with the glycans on glycoproteins present on the envelope of the virus, which are crucial for attachment or fusion, and prevent them from functioning normally in the replication cycle. The results of mechanistic studies on the antiviral action of NTL against the two different viruses (RSV and Flu A) probably suggest that during different phases of the viral infection cycle, the addition of NTL at the time of adsorption and/or penetration is very important. It is also worth noting that the modes of antiviral action of NTL against RSV and influenza A (H1N1) virus are significantly different. The inhibitory effect was mainly expressed in the early stage of the viral cycle in the H1N1 virus. Nevertheless, NTL actively inhibited RSV during the whole infection cycle of the virus.

It has been showed that *Urtica dioica* agglutinin (UDA) from *U. dioica* strongly binds to the glycans on the HIV-1 attachment protein gp120, and prevents HIV entry and transmission when co-cultivated with persistently HIV-infected and -uninfected cells in the lower μg/ml range (Balzarini 2006). Some other lectins are also able to inhibit viral entry. *Galanthus nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA) and UDA have been found to be able to inhibit the entry of HCV and HIV-1 into their target cells (Bertaux *et al.* 2007). Similarly, HHA inhibits SARS-CoV replication when added at the moment of infection (Keyaerts *et al.* 2007). This is in line with the mode of action of the influenza A virus in the present study. Nonetheless, HHA is also able to inhibit the extracellular viral load when added up to 5 h post-infection (a time near the end of the virus replication cycle). Thus, HHA interacts both at virus entry and at virus release (Keyaerts *et al.* 2007). The result of our time of addition experiment of NTL against RSV is in agreement with that of the interaction of HHA with SARS-CoV. NTL is active in inhibiting RSV during the entire infection cycle. To inhibit RSV infection, the possible targets that NTL can bind to are the glycoproteins present on the RSV envelope. Three envelope glycoproteins are present on RSV, including the G protein, F protein and SH protein (Feldman *et al.* 2001). Both the G and F proteins may be involved in the initial attachment of RSV to the host cell, and the F protein is largely responsible for the subsequent fusion process, though the highest level of fusion was observed when all three glycoproteins were present (Feldman *et al.* 2001). This interaction may be blocked if the RSV glycoproteins are bound by NTL, and thus the spread of RSV would be affected. In the present study, NTL was also found to be able to reduce plaque formation when added during the later infection phase. Whether NTL interferes with RSV

spread by binding to viral glycoproteins or inhibits other events during the process of viral replication or assembly remains to be elucidated.

Acknowledgements

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