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# Evaluating the use of Apo-neocarzinostatin as a cell penetrating protein

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**Protein–ligand complex neocarzinostatin (NCS) is a small, thermostable protein-ligand complex that is able to deliver its ligand cargo into live mammalian cells where it induces DNA damage. Apo-NCS is able to functionally display complementarity determining regions loops, and has been hypothesised to act as a cell-penetrating protein, which would make it an ideal scaffold for cell targeting, and subsequent intracellular delivery of small-molecule drugs. In order to evaluate apo-NCS as a cell penetrating protein, we have evaluated the efficiency of its internalisation into live HeLa cells using matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry and fluorescence microscopy. Following incubation of cells with apo-NCS, we observed no evidence of internalisation.**
*Keywords*: cell internalisation/cell-penetrating protein/drug delivery/Neocarzinostatin/protein–drug conjugate

## Introduction

The extensively studied neocarzinostatin (NCS) complex produced by *Streptomyces carzinostaticus* is a 12-kDa protein (Kuromizu *et al*., 1986) bound to an enediyne chromophore ligand (Napier *et al*., 1979). The enediyne chromophore is toxic (Kappen *et al*., 1980), which has been attributed to binding of the chromophore to DNA, upon which it induces double-stranded DNA cleavage (D'Andrea and Haseltine, 1978; Hensens *et al*., 1994). The SMANCS construct, in which NCS is conjugated to poly(styrene comaleic acid) (Maeda *et al*., 1979; Oda and Maeda, 1987) has been used clinically to treat hepatomas in humans (Konno, 1992; Konno *et al*., 1994).

The NMR (Takashima *et al*., 2005; Caddick *et al*., 2006) and crystal (Kim *et al*., 1993; Teplyakov *et al*., 1993) structures of NCS have been elucidated, which reveal a hydrophobic groove that binds the chromophore with nanomolar affinity (Povirk and Goldberg, 1980). The considerable stability of apo-NCS to reduction and denaturation (Meienhofer *et al*., 1972; Sudhahar and Chin, 2006), and its ability to bind a wide range of synthetic ligands have stimulated efforts to develop ligands for NCS for use as chemotherapeutics (Caddick *et al*., 2006). Such an activity could be coupled with the ability of NCS to functionally display complementarity determining regions loops from antibodies (Nicaise *et al*., 2004).

Analyses of DNA replication rates (Kappen *et al*., 1980) and gene transcription (Schaus *et al*., 2001) following incubation of live cells with holo-NCS suggest that holo-NCS is able to induce chromosomal DNA damage, and hence that the chromophore is able to enter the nucleus. The chromophore is highly unstable in its 'free' form, and so it has been suggested that the chromophore is protected from degradation in the cytoplasm by the NCS protein (Kappen *et al*., 1980). In addition, microscopy of live cells treated with fluorescently labelled holo-NCS suggests that holo-NCS is able to internalise, and accumulate in the nucleus (Takeshita *et al*., 1980; Oda and Maeda, 1987).

Given the favourable biochemical properties of apo-NCS, and the strong evidence for internalisation of holo-NCS, we have investigated the possibility that recombinant apo-NCS could act as a cell-penetrating protein, and therefore provide a platform for the targeted intracellular delivery of therapeutic cargo.

Here, we evaluate apo-NCS internalisation into live mammalian cells using two complementary techniques. Biotinylated- and rhodamine-labelled conjugates of two distinct cysteine mutants of apo-NCS were generated. Following incubation of HeLa cells with the relevant conjugate, matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry allowed quantitative analysis of intact apo-NCS whereas fluorescence microscopy allowed detection of apo-NCS that may be intact or proteolytically degraded.

## Methods

### *Protein expression*

NCS(wild-type) was subcloned from the vector p-CANTAB into pNIC28-Bsa4 using the primers TACTTCCAATCC ATGCTACAGGGAGCTGCTCCAACC and TATCCACCTT TACTGCTAGTTGAAGGAGATAGCAAC. NCS(+C) construct was generated by subcloning into pNIC28-Bsa4 using the primers TACTTCCAATCCATGCTACAGGGAGCTGCT CCAACC and TATCCACCTTTACTGTCAGCAACCACCA CCAGAGTTGAAGGAGATAGCAACGCC. The NCS(S14C) construct was generated by site-directed mutagenesis using the primers CATAAGCTGGAATACAATTTTAACTGCCACAAT GTTTACATCACCGCC and GGCGGTGATGTAAACATTG TGGCAGTTAAAATTGTATTCCAGCTTATG.

Non-isotopically labelled proteins were expressed in SHuffle cells (New England Biolabs) using the manufacturer's protocol. $^{15}$N–NCS(+C) was expressed in Shuffle cells in isotopic media, namely M9 salts with glucose (20 mM), $^{15}$N-ammonium chloride (0.1%), $CaCl_2$ (100 mM), $MgSO_4$ (2 mM) and Basal Medium Eagle vitamins (Sigma-Aldrich). All proteins were purified by nickel chromatography in the presence of β-mercaptoethanol (14 mM). The $His_6$ tag was removed by incubation with tobacco etch virus (TEV) protease for 2 h at 30°C, and apo-NCS was repurified by size-exclusion chromatography in water, using a HiPrep 26/60 sephacryl S-100 column. All subsequent modifications were completed at 4°C. Purified apo-NCS (150 μM) was reduced using tris(2-carboxyethyl)phosphine (TCEP, 200 μM) in sodium phosphate (50 mM, pH 8).

### *MALDI-TOF mass spectrometry*

All mass spectra were generated following addition of the sample to a saturated solution of sinapinic acid in $CH_3CN$ : $H_2O$ : $CF_3COOH$ (50 : 50 : 0.1). Each samples of 2× 1 μl were deposited and analysed using a Bruker Microflex Mass Spectrometer in positive linear ion mode. Spectra were accumulated over several hundred laser shots.

### *Protein conjugation*

Reduced apo-NCS (1 mg/ml) was labelled with biotin by incubation with EZ-Link Maleimide-$PEG_2$-biotin (Pierce, 500 μM) from a stock of EZ-Link Maleimide-$PEG_2$-biotin in water for 1 h. Excess labelling reagent was removed by diafiltration and the products were characterised by MALDI-TOF MS.

Reduced apo-NCS was labelled with tetramethylrhodamine (TMR) by incubation with of TMR-5(6)C2-maleimide (TMR-Mal, AnaSpec, 500 μM) from a stock of 20 mM TMR-5(6)C2-maleimide in dimethylformamide for 1 h. NCS-TMR was fully separated from free TMR-Mal by size-exclusion chromatography, using a HiPrep 26/60 sephacryl S-100 column in water. Products were characterised by MALDI-TOF MS.

### *Circular dichroism*

Circular dichroism (CD) spectra were recorded in 1-mm fused silica cuvettes using a Jasco J-715 spectrophotometer at room temperature in water, with 0.1 mg/ml of each construct. Thermal unfolding curves were obtained by monitoring the CD signal at 211 nm using 2-mm pathlength cuvettes and a heating rate of 1°/min.

### *Generation of Tat-TMR*

Tat-Cys (sequence H-RKKRRQRRRGC-$NH_2$, C S Bio, 500 μmol) was solubilised in degassed sodium phosphate (100 mM, pH8, 1 ml). Tat-Cys was reduced by addition of TCEP (500 μmol) and labeled by with TMR-5(6)C2-maleimide (1.5 mM), from a stock of 20 mM TMR-5(6)C2-maleimide in dimethylfomamide, for 1 h on ice. The product was concentrated by freeze-drying, followed by solubilisation in a minimum volume of 0.1% trifluoroacetic acid. The product was purified by high-performance liquid chromatography using a Vydac C18 (218TP) column, and freeze-dried. The product was characterised by MALDI-TOF MS.

### *Quantification of internalisation by MALDI mass spectrometry*

HeLa cells were seeded in sterile conditions in 24-well plates for 12 h before internalisation experiments. Sub-confluent cells ($10^6$ cells/well) were incubated for 1 h with NCS(S14C/+C)biotin (10 μM) in Hanks media. Cells were washed three times with Hanks media (1 ml), then treated for 5 min at 37°C with pronase (0.5 mg/ml, 200 μl) in sodium phosphate buffer (100 mM, pH 7.4). The digestion was halted by an addition of 100 μl 2× COmplete Mini Protease Inhibitors with EDTA (Roche) with BSA (0.1 mg/ml), and transferred to an eppendorf on ice. The cells were washed with of Tris-HCl buffer (50 mM, pH 7.4, 500 μl), and then with buffer A (1 ml of Tris-HCl buffer (50 mM, pH 7.4) containing 0.1% BSA); the cells were recovered at each stage by centrifugation for 2 min at 640 *g*. The pellet was resuspended in lysis solution (0.3% Triton X-100, 1 M NaCl, 150 μl) and the internal standard was added (0, 10 or 20 pmol 15N-NCS(+C)-biotin). The mixture was heated for 15 min at 100°C and then centrifuged for 5 min at 7080 g. The supernatant was mixed with of buffer A (850 μl) and incubated for 1 h with streptavidin-coated magnetic beads (Dynabeads M-280, Invitrogen, 10 μl). Subsequently, the beads were washed with buffer A (2 × 200 μl), buffer A containing 0.1% sodium dodecyl sulphate (2 × 200 μl), buffer A containing 1 M NaCl (2 × 200 μl), $H_20$ (3 × 200 μl), biotin (10 μM, 50 μl), $H_20$ (50 μl). Protein was eluted from the beads by incubation within a matrix of a saturated solution of sinapinic acid in $CH_3CN$ : $H_2O$ : $CF_3COOH$ (50 : 50 : 0.1; 3 μl) for 10 min at room temperature, then analysed by MALDI-TOF MS. Internalisation experiments were performed in triplicate and repeated at least twice independently.

For experiments without protease digestion, the cells were incubated and washed three times with Hanks media (1 ml), as described above. Lysis solution with internal standard (300 μl) was added, and the 24-well plate was placed in a boiling water bath for 15 min. The solution was then centrifuged and mixed with of buffer A (1700 μl) and incubated for 1 h with streptavidin-coated magnetic beads (10 μl). The beads were then treated as described above.

### *Fluorescence microscopy*

Concentrated NCS(S14C)-TMR, NCS(+C)-TMR or Tat-TMR were diluted to 10 μM concentration in Hanks media (Sigma-Aldrich). Adherent HeLa cells were incubated for 1 h at 37°C with the peptide or protein, followed by washing three times with Hanks media. Cells were then immediately imaged on a Deltavision fluorescence microscope using an Olympus 100X/1.40, Plan Apo objective, 0.035s exposure time with 10% transmission. z-sections were taken through the entire depth of the cells, and the images were deconvolved. An average intensity projection was generated, and brightness and contrast were adjusted equivalently for all images. At least 10 cells were observed in each condition, and 5 cells were imaged for each.

## Results and discussion

Apo-NCS contains four native cysteines that form two stable disulfide bonds, which are resistant to the commonly used

thiol-reducing agents (Meienhofer *et al*., 1972). We hence reasoned that by a using a point mutation strategy, we could introduce free cysteine residues into the NCS sequence that would allow facile and site-selective conjugation of either biotin or fluorophore labels. Apo-NCS was cloned into pNIC28-Bsa4, which introduces an N-terminal $His_6$ tag that can be cleaved by TEV protease. Two mutants with a single free cysteine were generated, namely NCS(S14C) (Ser to Cys mutation on loop 1, near the N-terminus), and NCS(+C) (addition of SGGGC at the C terminus) (see Supplementary data). These constructs were expressed in *Escherichia coli* and the $His_6$ tag was removed by TEV protease. Both constructs were reduced using TCEP, and fluorescently labelled with tetramethylrhodamine-maleimide to generate NCS(S14C)-TMR and NCS(+C)-TMR, or biotinylated with biotin-maleimide to generate NCS(S14C)-biotin and NCS(+C)-biotin.

CD spectra were obtained for each of the modified apo-NCS constructs described in this paper (Fig. 1). These spectra are consistent with previously published CD spectra for apo-NCS (Jayachithra *et al*., 2005; Sudhahar and Chin, 2006), Thermal denaturation (Fig. 2) confirms that the $T_m$ of the constructs are all between 61 and 66°C, and fully reversible. We hence conclude that apo-NCS remains stably folded at 37°C following introduction of a fifth cysteine residue, treatment with TCEP and introduction of biotin or tetramethylrhodamine. Analysis of these constructs by mass spectrometry confirmed the addition of either a fluorophore or biotin per protein molecule (see Supplementary data).

In order to determine how efficiently apo-NCS is internalised into mammalian cells, we adapted a strategy (Burlina *et al*., 2006) that has been used previously for quantifying the internalisation of small cell-penetrating peptides (CPPs). The method allows quantification of the amount of internalised CPP by adding a known amount of isotopically labelled CPP as an internal standard prior to analysis. The desorption/ionisation properties of the CPP and the isotopically labelled CPP are identical, which allows quantification and simultaneous detection by MALDI-TOF mass spectrometry. In this study, isotopically labelled $^{15}$N-NCS(+C)-biotin was prepared to quantify NCS cellular uptake (see Supplementary data). The mass spectrum shown in Fig. 3 is a 1 : 1 mixture

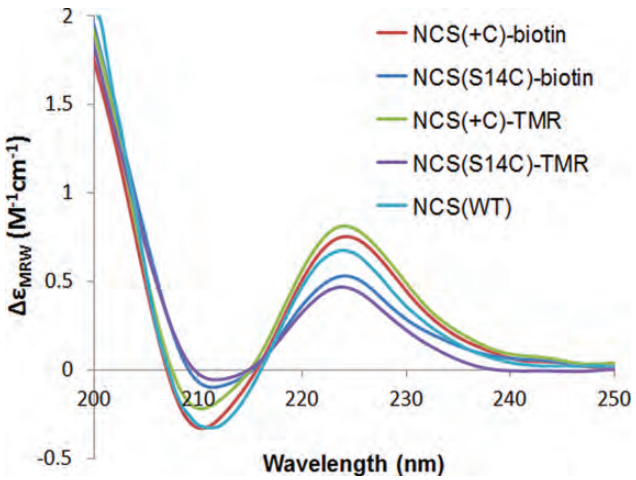


**Fig. 1.** CD spectra of unlabelled NCS(WT) and labelled NCS constructs.

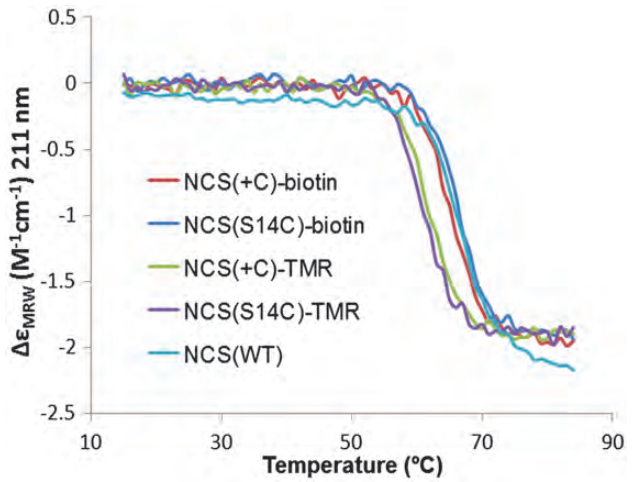


**Fig. 2.** Thermal denaturation curves of unlabelled NCS(WT) and labelled NCS constructs, monitored by CD.

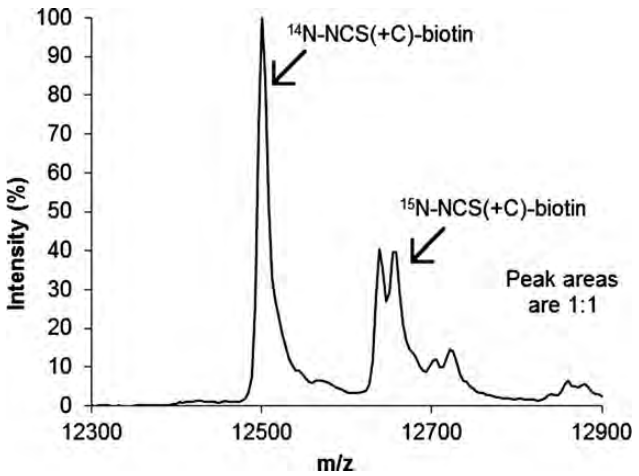


**Fig. 3.** MALDI-TOF mass spectrum of 1 : 1 $^{14}$N-NCS(+C)-biotin and $^{15}$N-NCS(+C)-biotin (10 pmol of each protein were mixed, then pulled down using streptavidin-coated magnetic beads).

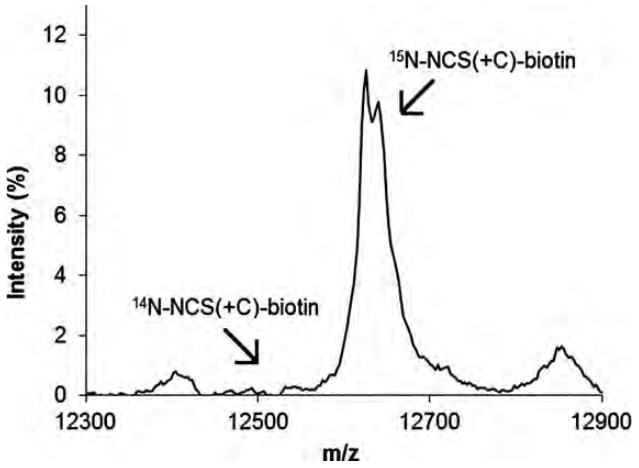


**Fig. 4.** MALDI-TOF mass spectrum of internalised NCS(+C)-biotin. [Cells were incubated with $^{14}$N-NCS(+C)-biotin (10 μM, 1 h at 37°C), then pronase (0.5 mg/ml, 200 μl). 15N-NCS(+C)-biotin (10 pmol) was added before lysis and pulldown of biotinylated species.]

of $^{14}$N-NCS(+C)-biotin and $^{15}$N-NCS(+C)-biotin. Partial hydrolysis of the maleimide (mass +18 Da), a known phenomenon under alkaline conditions,[22] was observed for the $^{15}$N-NCS(+C)-biotin construct. However, partial maleimide hydrolysis does not change the desorption/ionisation efficiency of the compound, as the peak area ratio is 1 : 1.

HeLa cells were first incubated with $^{14}$N-NCS(+C)-biotin. Conditions were employed that have previously been shown to permit internalisation of holo-NCS (Oda and Maeda, 1987). Cells incubated at 37°C are known to permit cell entry by endocytosis, pinocytosis, and direct translocation (Zorko and Langel, 2005). Mass spectra were obtained following the addition of internal standard ($^{15}$N-NCS(+C)-biotin) and subsequent to cell lysis. The internal standard was detected clearly on the mass spectrum, but no signal corresponding to intact $^{14}$N-NCS(+C)-biotin was observed (Fig. 4). No NCS intracellular digest was observed either but it should be noted that small biotinylated digests ($m/z <$ 1000) would be difficult to detect. This experiment suggested that apo-NCS was not internalised inside cells, but internalisation followed by rapid degradation could not be excluded.

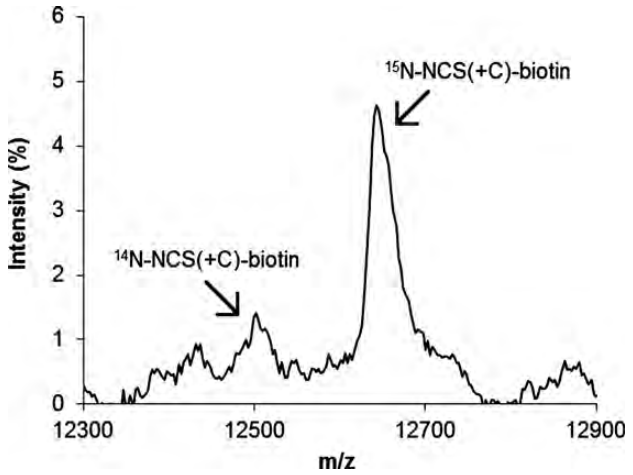


**Fig. 5.** MALDI-TOF mass spectrum of cell-associated NCS(+C)-biotin. [Cells were incubated with $^{14}$N-NCS(+C)-biotin (10 μM, 1 h, 37°C). $^{15}$N-NCS(+C)-biotin was added before lysis and pulldown of biotinylated species.]

To evaluate whether C-terminal biotinylation was inherently detrimental for cellular uptake, the experiment was repeated with apo-NCS mutant NCS(S14C)-biotin, biotinylated near the N-terminus. In order to minimise potential signal suppression effects, no internal standard was added. Again, no internalised apo-NCS was detected (see Supplementary data).

To detect any membrane-bound apo-NCS in addition to any internalised apo−NCS, the procedure was repeated, such that after incubation with $^{14}$N-NCS(+C)-biotin, the cells were lysed without pronase treatment. This eliminates any degradation of membrane-bound NCS by proteases. A small peak was observed at the expected mass for $^{14}$N-NCS(+C) biotin (Fig. 5). This peak is too small to quantify accurately, and may be background noise. Given the sensitivity of NCS detection by this technique, this suggests that less than 2 pmol apo-NCS is cell-associated following washing. The combined data suggest that apo-NCS neither tightly binds to nor internalises into HeLa cells.

We could not exclude from these experiments a rapid degradation of the internalised apo-NCS preventing its detection by MALDI-TOF MS (vide supra). To test this hypothesis, live HeLa cells were incubated with either fluorescently-labelled NCS(S14C)-TMR or NCS(+C)-TMR. The internalisation efficiency of NCS was compared with that of trans-activating transcriptional activator (Tat) from human immunodeficiency virus 1 (Vivès *et al.*, 1997), a peptide that is known to internalise relatively weakly compared with other CPPs, with an estimated intracellular concentration of 1 μM when incubated under similar conditions used in this experiment with CHO cells (Burlina *et al.*, 2005). As expected, Tat was internalised. In contrast, incubation with NCS(S14C)-TMR and NCS(+C)-TMR did not result in fluorescently labelled cells (Fig. 6) confirming that NCS does not enter cells. Using longer exposure images with higher illumination intensities (data not shown), it was clear that cells

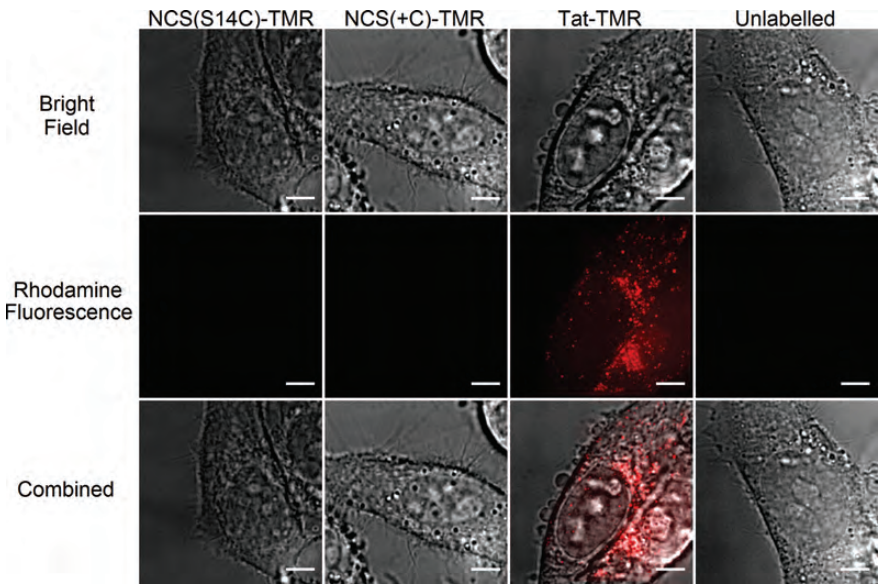


**Fig. 6.** Microscopy of HeLa cells treated with tetramethylrhodamine-labelled NCS (scale bars are 5 μm).

incubated with NCS(S14C)-TMR or NCS(+C)-TMR were often less fluorescent than the surrounding media.

These results clearly demonstrate that apo-NCS does not internalise into mammalian cells. Given that there is strong evidence for internalisation of holo-NCS, these results suggest that binding of the chromophore might be required for efficient internalisation to occur. This could be explained by a conformational change in the protein upon chromophore binding. A conformational change between the previously reported NMR structures of holo-NCS (1O5P) (Takashima *et al*., 2005) and apo-NCS (1j5H) (Urbaniak *et al*., 2002) is consistent with this hypothesis. The study on the effect of both natural and non-natural ligand binding on the internalisation of NCS into mammalian cells is on-going in our laboratory.

## Supplementary data

Supplementary data are available at *PEDS* online.

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