Mechanism of Free and Conjugated Neocarzinostatin Activity: Studies on Chromophore and Protein Uptake Using a Transferrin-Neocarzinostatin Conjugate

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Targeting studies using the anti-cancer agent neocarzinostatin (NCS), conjugated to antibodies have shown relatively poor specificity. From the literature, it is unclear whether NCS mediates its effects either in conjugated or unconjugated form. In the present work we have used a conjugating agent with transferrin, a biological ligand with a well defined endocytic route, to probe these mechanisms. NCS was covalently coupled to transferrin using the heterobifunctional reagent sulfo-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and 2-iminothiolane to give a stable thioether-linked conjugate with a ratio of 1.6 mol of NCS per mole of transferrin. The binding activity of transferrin was completely retained. Conjugation of NCS to transferrin resulted in an apparent enhancement of cytotoxicity. However, incubation with excess transferrin had no influence on the observed enhanced toxicity, indicating that endocytosis is not responsible.

Further experiments demonstrated that the apparent enhancement was dependent on incubation conditions and not an effect due to endocytosis of ligand. Studies where apo-NCS competed with holo-NCS and transferrin strongly indicated that the cytotoxicity of both NCS and conjugate is mediated by direct entry of the dissociated chromophore into the cell.

Introduction

Neocarzinostatin (NCS, mol wt: 11400 Da) is an antitumour protein antibiotic possessing a non-covalently bound molecule, which is the biologically active moiety, designated as chromophore in the literature because of its absorption at 340 nm. The protein moiety (apo-NCS) acts as a stabilizer and carrier of NCS-chromophore (Chr) (Napier et al., 1979; Napier et al., 1981; Kappen et al., 1980). The chemical structure of NCS-Chr has been elucidated by Edo et al. (1985) as a bicyclo[7,3,0]-dodecacadienediyne derivative having an α,β-N-methylfucosamine, 2-hydroxy-7-methoxy-5-methyl-1-naphthalene-carboxylic acid, ethylene carbonate and a highly strained epoxide. The chromophore is very sensitive to light and heat and in its isolated form it degrades very rapidly, especially at high pH and in the presence of reducing agents (Kap- pen and Goldberg, 1980, Edo et al., 1986). The mechanism of NCS interaction has been elucidated, revealing that the chromophore intercalates specifically between DNA base pairs and induces single-strand breaks and base release after activation to a biradical intermediate by reducing agents (Myers 1987; Myers and Proteau, 1989). Immunoconjugates with NCS have been investigated in targeting studies for potential cancer therapies. The rationale for these therapies is that by attaching a ligand to the drug, that the entry of drug into the cell will be controlled, allowing drug specificity to be achieved. Initial studies with NCS immunoconjugates suggested a useful specificity (Lüders et al., 1985, Takahashi et al., 1988), but more recent work using a wider range of antibodies and cell lines have shown a variable but low specificity between antigen positive and antigen negative cell lines (Gottschalk, 1990; Maibücher et al., 1994).

One possible explanation for these later results could be differences in rates and extents of uptake of the antibody ligands. Transferrin has also been proposed as a ligand for targeting to tumour cells,
and Kohgo et al. (1990), have reported a 9-fold enhancement of NCS cytotoxicity after coupling it to transferrin via a disulfide bond. The endocytosis of transferrin has been studied extensively and is well understood (de Jong et al., 1990), and Kohgo et al. (1990) demonstrated that transferrin-NCS (Tf-NCS) conjugates were taken up by the normal route for transferrin. We have carried out similar cytotoxicity experiments using Tf-NCS to help clarify the uptake of NCS immunoconjugate. In our experiments we have used a Tf-NCS conjugate linked by a non-reducible thioether bond to ensure the uptake of the intact conjugate.

Not only the mechanism of action of NCS immunoconjugates is uncertain. Different mechanisms have been proposed for the uptake of unconjugated NCS. Some results suggest a receptor-mediated endocytosis (Oda and Maeda, 1987; Oda et al., 1987) with the whole NCS molecule being internalized into the cell (Maeda et al., 1975). Other studies indicate that the apo-NCS does not necessarily need to cross the cell membrane for activity (Lazarus et al., 1977; Schor, 1989). A number of factors undoubtedly influence the mechanism or pathway by which NCS and Tf-NCS exert their cytotoxic effects. For example, previous reports on the NCS mechanism of action have shown that only a weak association exists between the NCS protein moiety and the cell surface (Gottschalk, 1990). Similarly, the association of the chromophore with its apoprotein is relatively weak under normal conditions of cell culture (Oda et al., 1987). Further experiments were therefore carried out to ascertain the contribution of these factors to the mechanism of NCS and NCS action.

**Materials and Methods**

**Preparation and isolation of apo-NCS**

A strategy for the quantitative isolation of apo-NCS, the non toxic protein component of NCS has been developed. First the NCS-chromophore was extracted in 3 steps with methanol from lyophilized NCS (crude preparation). The precipitated protein was then washed twice with methanol and subsequently dissolved in phosphate buffered saline (PBS). Further purification was performed by hydrophobic interaction chromatography (alkyl-superose) which resulted in a single peak fraction containing highly purified apo-NCS.

**Preparation of transferrin-NCS conjugate**

Human holo-transferrin (Tf) was purchased from Sigma (St. Louis, USA) and saturated with iron as described previously (Roskams and Connor 1990). Clinical-grade NCS generously provided by Kayaku Antibiotics (Tokyo, Japan) was used in this study. A twelve-fold molar excess of the cross-linking reagent, sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL, USA) was added to the NCS in 50 mM PBS, pH 7.2, and incubated for 90 min at room temperature. Transferrin was thiolated by treatment with a ten-fold molar excess of 2-iminothiolane in 50 mM PBS, pH 7.2, at 20 °C for exactly 30 min. Both proteins were immediately passed through a Sephadex G-25 column. A ten-fold molar excess of the SMCC linker-bearing NCS, SMCC-NCS, was reacted with the thiolated transferrin, SH-transferrin, in the dark overnight at room temperature. The Tf-NCS conjugate was separated from unbound NCS and transferrin dimers by gel filtration on a Superose 12 HR 16/50 column (Pharmacia) in 50 mM PBS, pH 7.2. The fractions containing the conjugate were pooled, concentrated and used in further experiments. The biological activities of native NCS and Tf-NCS conjugate were measured employing the fluorescence properties of the NCS-chromophore by the method of Gottschalk et al. (1990).

**Preparation of 3H-labelled transferrin**

Holo-transferrin has been labelled following a method described previously (Kummer, 1986). Briefly, 100 μg transferrin was incubated with a 1.5 fold molar excess of N-succinimidyl [2,3-3H] propionate (= 7.4 MBq) (Amersham) overnight at 4 °C. Excess radiolabel and cleaved N-hydroxysuccinimide were removed by gel filtration.

**Preparation of biotinylated transferrin**

Biotinylated transferrin was obtained by treating transferrin (10 μg/ml in PBS, pH 7.2) with a 20-fold molar excess of 100 mM solution of biotinamidocaproate-N-hydroxy-succinimide ester (Pierce, Rockford, IL, USA) in dimethylformamide. After 30 min incubation at 20 °C the product was purified by gel filtration on a Sephadex G25 column.
**Cell-ELISA**

To investigate the retention of binding affinity to the transferrin receptor after conjugating NCS to transferrin, a competitive ELISA using whole cells as the antigen target and biotinylated transferrin as the competitor was performed. For the cellular ELISA about 4 x 10^4 791T cells were applied to microtest plates per well and allowed to adhere by an overnight incubation and fixed with 2% paraformaldehyde. Then 50 µl of a solution containing 30 µg/ml biotinylated transferrin and either Tf-NCS conjugate or free transferrin, respectively, were added in various concentrations to each well. To enhance the sensitivity of the assay, preformed complexes consisting of streptavidin and biotinylated horseradish peroxidase (both Boehringer, Mannheim, Germany) were applied to the 791T cells for 1h, followed by a treatment with a solution of 2,2'-azino-di-3-ethylbenzthiazoline (Boehringer) with a trace of H_2O_2 for 30 min. The absorbance at 410 nm was a measure of the quantity of bound biotinylated transferrin.

**Estimation of the number of transferrin receptors**

791T Cells (2 x 10^5) were plated out in microcentrifuge vials and incubated on ice for 60 min with suitable dilutions of tritium-labelled transferrin (specific activity 82 GBq/mmol). The resultant cell-bound radioactivity was determined following three successive washing and centrifugation steps. The supernatant was collected and counted separately. The number of receptors and the K_D-value was calculated from a Scatchard Plot.

**Cytotoxicity assay**

Osteogenic sarcoma 791T cells (1 x 10^4/well) were incubated in DMEM medium (Gibco/BRL, Eggenstein, Germany) (without serum) in microtest plate (Nunc, Roskilde Denmark) with NCS or Tf-NCS in a concentration range of 0 to 3 µg/ml (expressed as the amount of active NCS), followed by a recovery phase in medium supplemented with 10% fetal calf serum (FCS) over a period of 46 h. The cells were then treated with 3.7 kBq of [3H]leucine (specific activity, 2.11 TBq/mmol) added to each well. After a further 6 h, the cells were harvested onto glass filter membranes (Dunn, 934 AG Filter, Asbach, Germany) using a PHD cell harvester (Cambridge Technology Inc., USA) and the radioactivity was counted. Cytotoxicity has therefore been estimated by the inhibition of [3H]leucine incorporation into protein, and is defined as the molar concentration giving 50% inhibition of leucine incorporation (IC_50).

In a variation of this method the cells were incubated with either NCS or Tf-NCS conjugate in medium with serum for 48 hours. Serum was added to maintain viability of the cells during the prolonged incubation period. The cells were then treated with [3H]leucine following the same procedure as described above.

**Competitive cytotoxicity assay**

Competitive toxicity tests have been performed applying a constant amount of drug, 3 µg/ml NCS or 1.5 µg/ml Tf-NCS, respectively, with increasing amounts (81-fold) of apo-NCS. The cells were incubated in medium without serum for one hour, following the same procedure as described above. In a variation of this method the cells were incubated with a constant amount of either free NCS or Tf-NCS conjugate supplemented with increasing amounts of transferrin (80-fold), for 48 h in medium with serum.

**Results**

**Characterization of the transferrin-NCS conjugate**

Chemical conjugation not only yields Tf-NCS conjugates, but also leads to the formation of dimers and oligomers in minor quantities. Crosslinking was minimized by applying a large molar excess (ten fold) of NCS. The TF-NCS conjugates were separated from these side products by gel filtration. The iron saturation of the conjugated transferrin was determined photometrically (λ = 465 nm) to be 90–95%. Since different levels of substitution in Tf-NCS conjugate were expected, the product was analysed with a SDS-PAGE using the Pharmacia Phast-System. The result indicated that the conjugate prepared by our method carried up to 4 mol of NCS per mole of transferrin. Gel scanning analysis with a LASER-densitometer (Ultrascan XL, Pharmacia LKB, Freiburg Germany) revealed that the major components of the product were Tf-(NCS)_1, Tf-(NCS)_2 and unchanged transferrin, whereas Tf-(NCS)_3 and Tf-
(NCS)$_4$ were of negligible concentrations (Fig. 1). An average of 1.6 mol NCS was bound to each transferrin molecule. However, the conjugate was accompanied by residual unmodified transferrin, which amounted to approximately 20%. The separation of this portion by gelfiltration was not feasible due to the relatively small difference in size.

**Binding affinity to the transferrin receptor**

Since binding of NCS to transferrin may lead to an alteration of its physical properties, the binding affinity of the conjugate was compared with that of free transferrin. A cellular ELISA was performed, applying various concentrations of Tf-NCS conjugate and free transferrin, respectively, competing with a constant amount of biotinylated transferrin in a concentration found to give saturation of the receptor (data not shown). It was found that the conjugate retained almost all of its binding affinity to the transferrin receptor (Fig. 2). The number of transferrin receptors presented on a 791T cell was estimated in a separate experiment to be $3.8 \times 10^5$ per cell with an equilibrium constant $K_d = 2.0 \times 10^{-7}$ m by Scatchard analysis.

**Cytotoxicity assay**

The cytotoxicity of Tf-NCS conjugate and native NCS was determined using the osteogenic sarcoma cell line 791T by inhibition of $[\text{H}]$leucine incorporation into protein. When the cells were continuously exposed to the agents over a period of 48 h in medium with 10% FCS, the conjugate was about 5 fold as toxic as native NCS. The 50% inhibitory concentration ($IC_{50}$) values of conjugate and NCS were 300 ng/ml and 1500 ng/ml respectively (Fig. 3). This experiment appears to suggest that cytotoxicity of NCS conjugates may involve receptor binding and endocytosis. However a competitive cytotoxicity assay using excess free transferrin (up to 80 fold) to compete with the conjugate did not result in a decrease in toxicity (data not shown).

Changing the incubation conditions of the cytotoxicity assay led to a different result. After a one hour incubation of conjugate or NCS in DMEM without serum followed by 46 h in medium containing 10% FCS, Tf-NCS and native NCS had identical cytotoxic effects (Fig. 4) with an $IC_{50}$ value of 700 ng/ml. A further experiment has been performed under the original conditions using the lysosomotropic agent ammonium chloride (10 mM) in a 48 h incubation in medium with 10% FCS. The resulting increase in the pH of the endosomal compartment during the incubation period had no effect on the toxicities of either free or conjugated NCS ($IC_{50}$ 1500 ng/ml and 300 ng/ml, respectively, Fig. 3).

From these experiments, the transferrin endocytic pathway appeared to contribute little if at all, to the cytotoxicity of the Tf-NCS conjugate. Similarly the question of the normal mode of NCS activity became very important in interpreting these results. Two mechanisms have been proposed for the action of NCS. Firstly, that a receptor exists for
NCS concentration [ng/ml]

Fig. 3. $[^{3}H]$leucine incorporation assay with Tf-NCS conjugate (●) and free NCS (○) on 791T cells with an incubation time of 48 h in medium with serum. The assay has been repeated in the presence of 10 mM NH$_4$Cl (dotted lines). Untreated cells (0% toxicity) have incorporated the labeled leucine in an order of 10,000 to 15,000 DpM.

Fig. 4. $[^{3}H]$leucine incorporation assay with Tf-NCS conjugate (●) and free NCS (○) on 791T cells with an incubation of 60 min in medium without serum. A pulse of label was added after a further 48h incubation in medium with serum.

Inhibition of $[^{3}H]$Leu incorporation

Ratio: competitor : NCS

The protein moiety, but with a very low binding affinity (around 10$^{-4}$-10$^{-5}$) (Oda and Maeda, 1987). Alternatively, that the free chromophore is able to pass through the plasma membrane and diffuse across the cell to interact with DNA directly.

To investigate these possibilities, 791T cells were incubated for 1h in medium without serum with 3000 ng/ml of NCS and coincubated with increasing amounts of either apo-NCS or BSA as a control. As expected in the case that apo-NCS competes with the active species for putative receptors, cytotoxicity was decreased (Fig. 5). As shown in the same figure, coincubation of Tf-NCS (1h, in medium without serum) conjugate with excess apo-NCS led to a reduced cytotoxicity as well. However, pre-incubation of cells with apo-NCS at 4 °C to block putative cell surface receptors prior to exposure to active NCS (3 µg/ml) did not result in a decrease of toxicity, even though the ratio of apo- to holo species was up to 80-fold (data not shown).

To determine whether endocytosis of intact NCS is required in its normal mode of action, 791T cells were incubated with NCS in serum at 4 °C and washed. Despite the fact that endocytosis is prevented under these conditions, a considerable cytotoxic effect was seen (Fig. 6). The incubation period, however, had to be extended to 2 hours to achieve sufficient cell killing, as may be expected from the effect of temperature on physical and chemical processes.

Discussion

To investigate how NCS mediates its effects in the conjugated form, a transferrin conjugate has been synthesized, to take advantage of the well documented transferrin endocytic pathway. The composition of this conjugate is therefore very similar to that reported by Kohgo et al. (1990).

Cells incubated continuously with Tf-NCS conjugate over a period of 48 h in medium with 10% serum, resulted in a 5-fold higher activity than free NCS under the same conditions.

In Kohgo's work (Kohgo et al., 1990) a 1% serum supplement was used. Our cell lines will not tolerate these low serum conditions so we carried
Fig. 6. Incubation of 791T cells with NCS in medium without serum for two hours at 4 °C to prevent endocytosis. The cells were then washed three times and allowed to recover for 48 hours in medium with serum.

out this experiment in 10% serum. The number of transferrin binding sites and the equilibrium constant of 791T cells were estimated and compared with the corresponding values for K562 cells (Klausner et al., 1983) which were used by Kohgo et al. (1990). The number of binding sites of 791T cells are about twice as high as found for K562 cells, whereas the equilibrium constant is considerably (50×) lower. The equilibrium constant for 791T cells is similar to a value reported for HeLa cells (K₅ = 3.4 × 10⁻⁷ M) (Ward et al., 1982). Our result is therefore similar to the data reported by Kohgo et al. (1990) where a 9-fold selectivity was reported, and consistent with their explanation that the binding and internalisation of transferrin may be responsible for the apparent selective effect. However, competition with a large excess of free transferrin did not decrease the cytotoxicity observed, strongly indicating that another explanation is more likely.

Changing the assay conditions to a 1 h incubation of the agents in medium without serum, followed by a 46 h recovery period in medium containing 10% FCS abolished the increase in Tf-NCS activity. Under these conditions both NCS and Tf-NCS showed identical cytotoxicities.

Conjugation of NCS to macromolecules has however been reported to increase the stability of NCS, and this extra stability may explain the increase in cytotoxicity of the conjugate with the initial co-incubation period in the presence of serum (Gottschalk et al., 1991). An alternative possibility of increased conjugate activity concerns the receptor mediated internalisation of transferrin. During endocytosis the transferrin conjugate will pass through the acidic endosomal compartment. NCS is known to be more stable in acidic media, and this effect could also contribute to differences in cytotoxicity during the initial co-incubation period in the presence or absence of serum. This possibility was examined by the determination of activity in the presence of the lysosomotropic agent ammonium chloride which is known to raise the pH of the endosomal and lysosomal compartments. This treatment had no effect on the toxicity of either free or conjugated NCS, thus also excluding an effect of endocytosis increasing the stability and consequently the potency of NCS and Tf-NCS conjugates.

Further investigations were made to distinguish between the two proposed mechanisms of NCS activity. 1) A receptor mediated endocytosis of NCS as proposed by Maeda et al. (1975) and 2) diffusion of dissociated chromophore through the cell membrane and cytoplasm to the nucleus (Lazarus et al., 1977; Schor, 1989).

Experiments were carried out to investigate the effects of the presence of excess apo-NCS on the toxicity of the active species. A reduced activity was found, which is consistent with a receptor recognition, as has previously been reported (Oda and Maeda, 1987; Oda et al., 1987; Maeda et al., 1975). However a specific cell surface receptor for NCS is unlikely as we were unable to decrease cytotoxicity by pre-incubating cells with apo-NCS at 4 °C to block putative binding sites prior to exposure with active NCS.

The biological activity of NCS was also seen when incubated with cells at 4 °C to prevent endocytosis, followed by washing. This experiment taken together with the previous experiment, strongly supports the hypothesis that endocytosis is not a necessary route for unconjugated NCS activity, and would therefore suggest that it is also unnecessary in the case of a Tf-NCS conjugate.

The reduced activity of NCS on incubation with an excess of apo-NCS is also consistent with a scavenging effect of apo-protein on dissociated free chromophore outside the cell. Interestingly coincubation of Tf-NCS conjugate with excess apo-NCS also led to a reduced cytotoxicity. Scav-
enging of the chromophore by apo-NCS is therefore the most likely explanation as it is consistent with both the NCS and Tf-NCS conjugate results.

To distinguish more clearly between these two possible uptake pathways, we have investigated the data on the excess of apo-NCS on the inhibition of cytotoxicity more closely. If the dissociated free chromophore is the active principal in crossing the cell membrane, cytotoxicity of both free NCS and Tf-NCS should be proportional to the concentration of free chromophore in the medium. Alternatively if the mechanism of NCS is related to the binding and internalisation of the putative NCS protein receptor, the cytotoxicity should be related to the receptor occupancy by (active) holo-NCS. These equilibria can be calculated by application of the law of mass action.

1) Considering the dissociated free chromophore concentration. When NCS is in solution, chromophore exists in equilibrium with its apoprotein.

\[ K = \frac{[\text{Apo}] \cdot [\text{Chr}]}{[\text{Halo}]} = 10^{-10} \text{ M} \]

Addition of excess apoprotein will change the equilibrium in favour of holo-NCS reducing the concentration of free chromophore. From the known equilibrium constant of \(10^{-10} \text{ M}\) (Napier et al., 1979) and by transforming this equation, the concentration of free chromophore can be calculated at any given ratio of holo-NCS and apo-NCS.

\[
\begin{align*}
[A\text{po}]_{\text{total}} &= [\text{Chr}]+[A\text{po}]_{\text{added}} \\
[\text{Holo}] &= [\text{Holo}]_0 - [\text{Chr}] \\
\Rightarrow K &= \frac{([\text{Chr}]+[\text{Apo}]_{\text{add}}) \cdot [\text{Chr}]}{[\text{Holo}]_0 - [\text{Chr}]} 
\end{align*}
\]

When the calculated concentration of dissociated free chromophore was compared to the inhibition of cell growth (leucine incorporation) obtained experimentally in the competition assay with excess apo-NCS, a strong correlation was obtained (Fig. 7).

2) To calculate the receptor occupancy. If we assume that apo-NCS binds to the receptor in a similar manner to holo-NCS, we can again use the law of mass action to calculate the equilibrium between free NCS, free receptor and NCS-receptor complex (NCS-R) applying the equilibrium constant found by Oda and Maeda (1987).

\[ K = \frac{[\text{NCS}] \cdot [\text{R}]}{[\text{NCS-R}]} = 10^{-4} - 10^{-5} \text{ M} \]

Thus an increase of NCS concentration will lead to a shift of the equilibrium towards the formation of the NCS-receptor complex. Under the assumption that apo-NCS binds to the receptor with the same avidity as holo-NCS, additional apo-NCS will also lead to an increased formation of the NCS-receptor complex. However, only the active species bound to the receptor will contribute to the cell killing activity. The portion of protein bound to the receptor containing an active chromophore is proportional to the ratio of holo-/total NCS prevalent in the solution.

The concentration of NCS-receptor complex in a solution with given holo-/apo-NCS ratio can be calculated by introducing \([\text{NCS}]_0\) and \([\text{R}]_0\) as initial concentrations of NCS and receptor, respectively.

\[ \text{Inhibition of } 3^\text{H}\text{-Leu incorporation} \]

Fig. 7. Correlation between cytotoxicity and the diffusion uptake model. Using data from Fig. 5. Inhibition of \([^3\text{H}]\text{Leu-}
incorporation (---o---, right hand vertical axis) is plotted against total NCS-protein concentration (initial 3000 µg/ml holo-NCS plus different ratios of apo-NCS as inhibitor). The observed cytotoxicity is compared to the calculated concentration of free NCS chromophore (---, left hand axis).
\[
[NCS] = [NCS]_0 - [NCS-R] \\
[R] = [R]_0 - [NCS-R] \\
\Rightarrow K = \frac{([NCS]_0 - [NCS-R]) \cdot ([R]_0 - [NCS-R])}{[NCS-R]}
\]

In the above formula \([NCS-R]\) represents the concentration of the NCS-receptor in the equilibrium but does not take into consideration whether the bound NCS contains chromophore or not. The proportion of \([NCS_{\text{active-R}}]\) to \([NCS_{\text{inactive-R}}]\) is directly proportional to the ratio of holo-/apo-NCS in the solution. Plotting the calculated amount of active NCS bound to receptor against toxicity (inhibition of leucine incorporation) for the same experiment as presented in Fig. 7, we obtain Fig. 8 showing a very poor correlation between receptor binding of active holo-NCS and cytotoxicity. Furthermore, when a transferrin conjugate is used instead of unconjugated NCS, the strength of binding of transferrin to its receptor is much greater than that of NCS (100 fold), and transferrin receptor effects should overwhelm any binding and competition effects between NCS and their receptors. In short we would expect to see no inhibition of transferrin conjugates by an excess of apo-NCS if internalisation was important to the mechanism of action of NCS.

However, when Tf-NCS conjugates competed against excess apo-NCS, a similar result was obtained to that found with unconjugated NCS. When these results are transformed, there is still a proportional relationship between free dissociated chromophore and cytotoxicity over the central part of the activity range for both conjugates, as shown in Fig. 9. The slope of the line produced by the transferrin conjugate competing with scavenging apo-NCS was different to that of NCS. Additionally, the slope of the line for the transferrin conjugate varied according to the incubation time of the apo-NCS conjugate (data not shown). These differences in the slope of the Tf-NCS assay in comparison to NCS can be accounted for by the greater stability of conjugated NCS which has been previously demonstrated with antibody conjugates (Gottschalk et al., 1991).

Taken together, these studies have looked in detail at the question of the involvement of endocytosis of the protein moiety in the mechanism of action of NCS and NCS-conjugates. Despite many experiments we have been unable to find evidence
which supports a significant contribution of endocytic pathway to the mechanism of action of NCS. The relationship shown in this paper between free chromophore concentration and cytotoxicity strongly suggests that extracellular release of the free chromophore and diffusion is the most likely pathway of chromophore uptake. Conversely we have found little or no support for the idea that a low affinity "receptor" contributes to the mechanism of NCS action.

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