INCREASED SERUM STABILITY AND PROLONGED BIOLOGICAL HALF-LIFE OF NEOCARZINOSTATIN COVALENTLY BOUND TO MONOCLONAL ANTIBODIES

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The pharmacokinetics of neocarzinostatin (NCS) have been compared to NCS conjugates with monoclonal antibodies using Balb/c and tumor bearing nude mice. Data on blood and whole body clearance revealed that the high MW conjugate persists in the body far longer and at a higher level than the free drug. Excretion of the free drug occurs with an extremely rapid renal clearance and localization of the remaining drug in the kidney, whereas the NCS immunoconjugate remained in circulation far longer allowing time for tumor localization to occur without renal accumulation of drug.

In addition, NCS conjugated to monoclonal antibody was found to retain its activity in human serum better than free drug, in agreement with data obtained for other NCS-derivatives. Half-time of inactivation was greatly extended when measured under relevant conditions in a DNA strand-break assay.

The results indicate that two of the most important requirements for the successful targeting of NCS *in vivo*, decreased clearance rate and increased serum stability are achieved by conjugation to antibody. Both results increase the probability of NCS accumulating in tissue while still in its active form.

Coupling of NCS to monoclonal antibody decreases clearance and inactivation rate and increases localization of the active drug in tumor tissue.

The antitumor antibiotic neocarzinostatin (NCS) has been used in clinical trials for the chemotherapy of human cancers in recent years.[1,2] It is evident, however, from both *in vitro* and *in vivo* experiments, that both pharmacokinetics as well as serum stability of NCS are disadvantageous. The drug exhibits an extremely rapid renal clearance and becomes inactivated in serum with a half-life in the order of minutes.[2~4]

The drug consists of an apoprotein and a non covalently associated chromophore that is very sensitive to light and heat.[5] The primary structure of the apoprotein has been elucidated and revised,[6] and the binding site involved in the association of the non-protein chromophore as well as the regions for hydrophobic interactions have been described.[7] It was noted that the only two amino-groups present in the protein (one α-amino-group at Ala 1, one ε-amino-group at Lys 20) are not involved in the cytotoxic action and therefore modification at one or both of these functionalities results in no loss of biological activity.[8] Conjugates of NCS and a variety of markers such as fluorescein,[9] rhodamine or biotin (GOTTSCHALK, U.; A. MAIBÜCHER and K. TRUTSCHLER-EBERT; unpublished data), peroxidase,[10] SMA,[11] succinyl,[12] transferrin[13] and monoclonal antibodies[14] have been prepared *via* the NH_2-groups in studies on NCS and its possible role in tumor therapy. In the present study the pharmacokinetics and serum stability of conjugates of NCS and the monoclonal antibody 791T/36 have been examined. This has

included the assessment of blood and whole body levels in Balb/c mice and the resulting biodistribution as well as stability data in human serum. The monoclonal antibody has itself been extensively characterised in tumor localization and pharmacokinetic studies in human xenografts making it an appropriate antibody for this study.[15]

Materials and Methods

Clinical NCS generously provided by Kayaku Antibiotics (Tokyo, Japan) was used in this study.

BW431/26 was kindly provided by Dr. RICHARD BAUM from the University Hospital in Frankfurt, Germany.

Conjugates have been synthesized with the monoclonal antibody 791T/36 raised against the osteogenic sarcoma cell line 791T[16] and the anti-CEA monoclonal antibody BW431/26[17] *via* the heterobifunctional reagent *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)[18] resulting in serum stable, non-reducible thioether linkages.

The products were chemically and biologically characterized and stored in PBS-buffer, pH 6.5 at 4°C.

For the measurement of active NCS-concentration in serum, samples were subjected to a DNA strand-breaking assay in which the strand-break introduction in Col E1 plasmid DNA was determined according to the method of MENKE *et al.*[19] Briefly, NCS and antibody-NCS-conjugates were incubated in freshly prepared human serum at a NCS-concentration of 1 μg/ml. After reaction for 10 or 60 minutes, samples of the mixture were incubated in NaCl 60 mM, Na-citrate 6 mM, Tris-HCl 15 mM and dithiothreitol 0.15 mM at pH 7.5 and 37°C. 20 μl aliquots were withdrawn at various time points and mixed with ^{3}H-labeled Col E1 plasmid DNA, at an end concentration of 1～2 μg/ml. Retention of strand-breaking activity has been calculated from the loss of supercircular forms after agarose gel electrophoresis according to POVIRK *et al.*[20]

The conjugates and free NCS were labeled with ^{125}I by the iodogen method to a specific activity of 7×10^{7} cpm/mg.[21]

Balb/c mice were from Bantin and Kingman, Hull, UK, and nude MFI-nunu/ola mice from Harlan Olac, Oxon, UK. Nude mice were housed under sterile conditions.

791T xenografts were subsequently passaged sc in nude mice by grafting small pieces of tumor, and allowed to grow for 10～14 days before use.

To determine blood clearance and whole body clearance of free drug and conjugate, animals received a single injection of 100～200 μl protein-solution (about 0.5 mg/ml) *via* the tail vein. Blood clearance data were obtained from blood samples drawn at various time points. Whole body clearance data were obtained by counting whole mice in a 75 × 75 mm well scintillation counter (John Caunt, Oxford, UK) at appropriate time points.

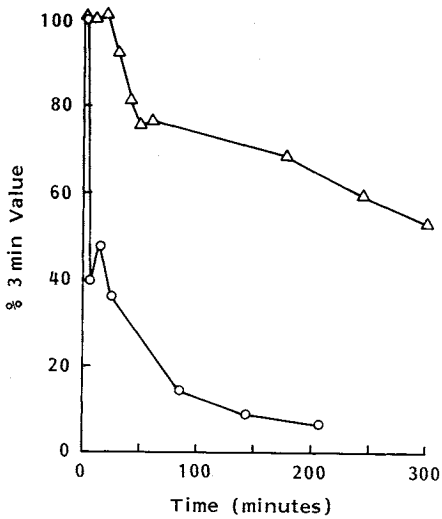
Results

Blood clearance studies over a short (6 hours) period (Fig. 1) show that the clearance of NCS from the blood is extremely rapid with only 5% of drug remaining after 4 hours. However, conjugation to the larger IgG macromolecule greatly reduces the rate and amount of clearance. Blood clearance of the conjugate is however, still slightly faster than would be expected for unconjugated antibody. Looking at the whole body clearance over a longer period (Fig. 2), gives a better idea of the possible therapeutic advantages of conjugated NCS. It can be seen that NCS is not only eliminated rapidly from the blood but that it is mostly rapidly cleared from the body by 6 hours. However, a small residual amount of drug remains even out to 73 hours. In contrast, conjugated NCS has a rapid initial clearance over the first 6 hours, but then settles to a slower rate which still leaves 20% of the initial dose retained in the body after 73 hours.

Parallel biodistribution studies show where the drug is capable of exerting its cytotoxic action. At 6

Fig. 1. Blood clearance of ^{125}I-NCS and ^{123}I-[791T/36-NCS] from Balb/c mice, expressed as mean % injected dose.

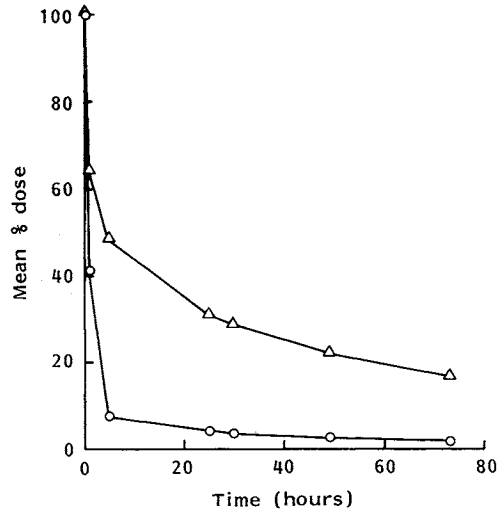
○ ^{125}I-NCS, △ ^{123}I-[791T/36-NCS].



10 μl serum samples were drawn after different time points. Concentrations of protein in blood samples taken 3 minutes after iv injection were considered as the maximum level (100 %). Administered doses were 1.25×10^7 cpm/mouse for NCS and 4×10^6 cpm/mouse for conjugate. Mean of 3 and 4 animals, respectively.

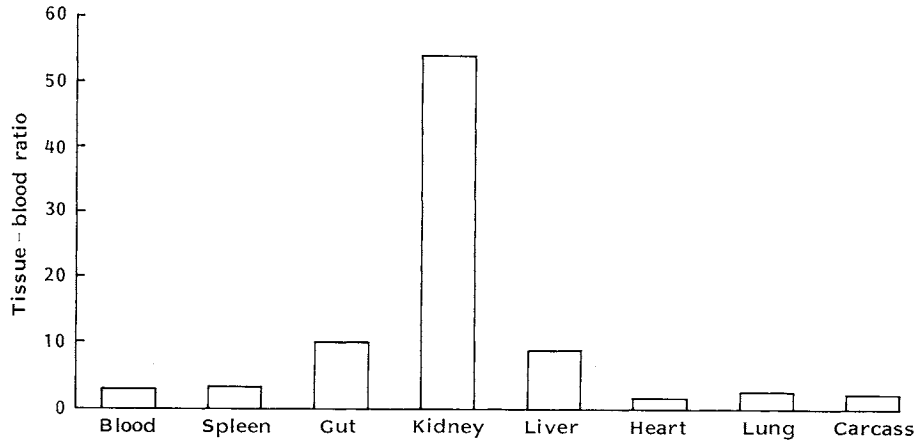
Fig. 2. Whole body elimination of ^{125}I-NCS and ^{125}I-[791T/36-NCS] from Balb/c mice, expressed as mean % injected dose.

○ ^{125}I-NCS, △ ^{125}I-[791T/36-NCS].



10 μl serum samples were drawn after different time points. Mean administered doses were taken as the maximum level (100 %). Administered doses were 2.5×10^5 cpm/mouse for NCS and 1.8×10^5 cpm/mouse for conjugate. Mean of 2 and 5 animals, respectively.

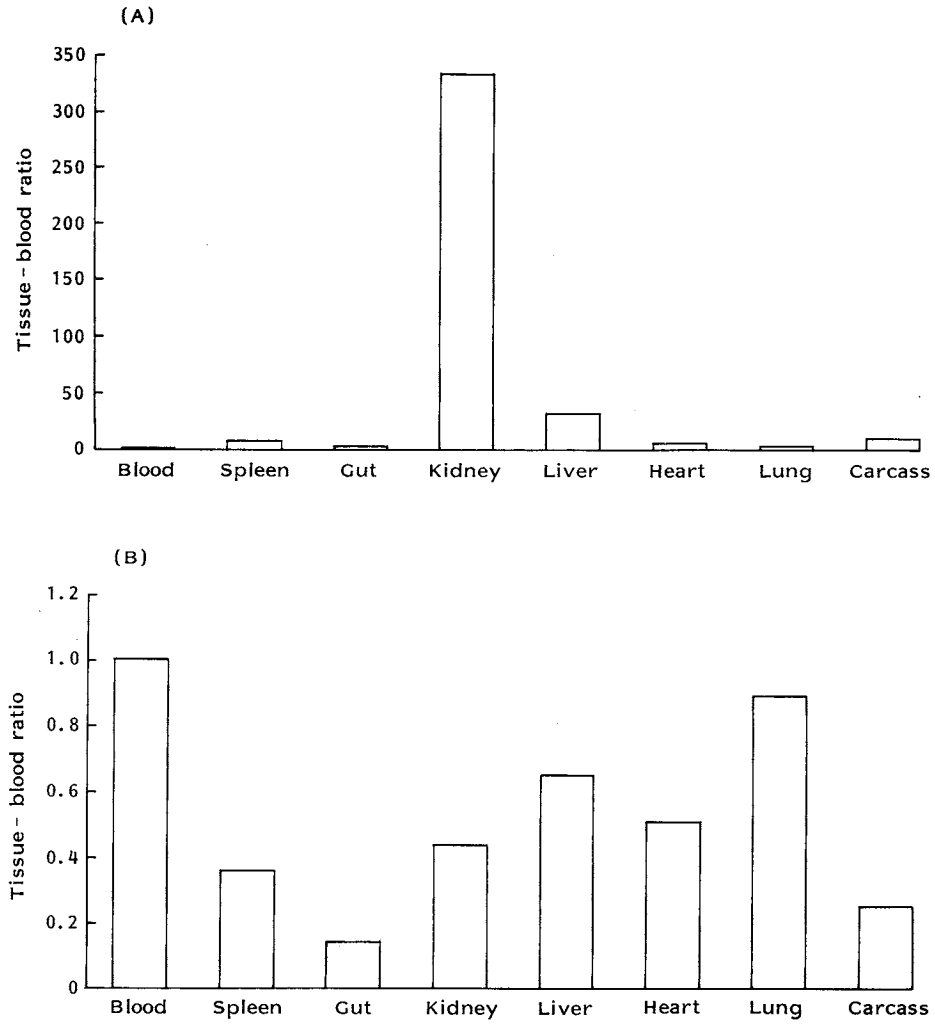
Fig. 3. Biodistribution of ^{125}I-NCS in Balb/c mice at 6 hours.



Animals received an iv administered dose of 1×10^6 cpm. Mean of 4 mice.

hours, when NCS has largely been eliminated from the blood, the highest relative concentration of NCS is found in the kidney where presumably it accumulates as a result of renal excretion (Fig. 3). Some localization also occurs in both gut and liver. The renal and liver uptake is expected since these are known sites of toxicity.[2] After 73 hours (Fig. 4A) NCS is still sequestered in kidney and some in liver, but amounts of drug in other organs are extremely low. In contrast the biodistribution of conjugate at this time (Fig.

Fig. 4. Biodistribution of (A) ^{125}I-NCS and (B) ^{125}I-[791T/36-NCS] in Balb/c mice at 73 hours.



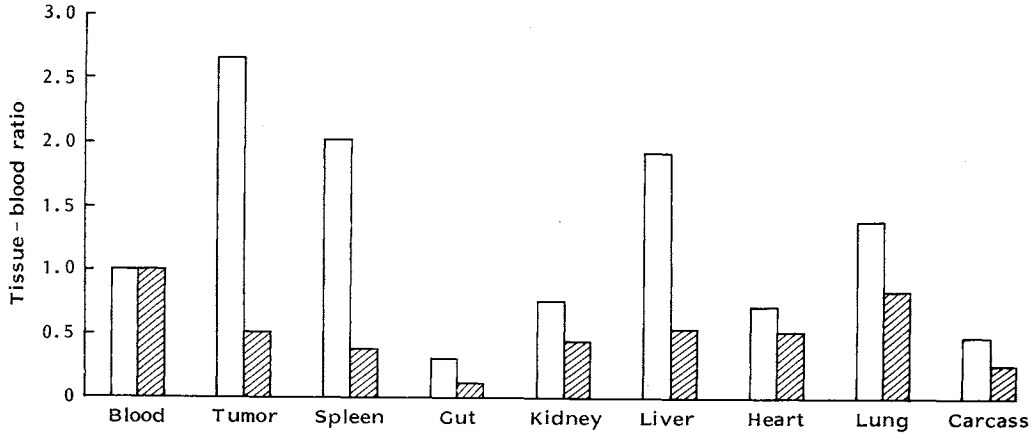
Animals received an iv administered dose of 2.5×10^5 cpm (A) and 1.8×10^5 cpm (B), respectively. Mean of 2 and 5 mice, respectively.

4B) is more a reflection of the pattern of localization seen with 791T/36 antibody.[22] Renal localization is not prominent. The time of 73 hours was chosen for this comparison because it is the optimum time to see a good specific localization of antibody.

We also report preliminary data on the specific localization of NCS conjugate in tumor bearing nude mice (Fig. 5). In this experiment groups of mice bearing 791T osteogenic sarcoma xenografts were treated with either the specific 791T/36-NCS conjugate or the non-specific BW431/26-NCS conjugate for a period of 36 hours. Biodistribution at this time point showed that tumor localization of the specific conjugate occurred. High activity was also seen in spleen, gut and lung tissue. This localization has also been seen with free antibody in large tumors in this system and is thought to be due to formation of immune complexes with minute amounts of circulating antigen. This effect would be particularly apparent in this sort of experiment where only a small amount of labeled conjugate is injected. In contrast the

Fig. 5. Biodistribution of specific ^{125}I-[791T/36-NCS] and non-specific ^{125}I-[BW431/26-NCS] conjugates in 791T tumor bearing nude mice at 36 hours.

Open bars: Specific conjugate, closed bars: non-specific conjugate.



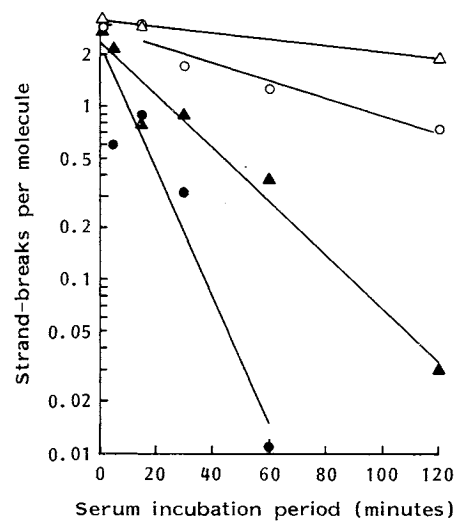
Administered doses were 1.46×10^7 cpm/mouse for the specific and 2.06×10^7 cpm/mouse for the non-specific conjugate. Mean of 2 and 3 mice, respectively.

non-specific antibody showed no specific localization in any tissue, all tissue to blood ratios being less than one.

To complement these localization studies and assess their relevance to a therapeutic situation, experiments have also been carried out on the stability of NCS in serum. NCS has been reported to be relatively unstable in serum, but these experiments have been carried out with non-physiological amounts of drug in diluted serum.[3,4] The results presented in Fig. 6 show the course of inactivation of NCS as well as 791T/36-NCS conjugates in fresh human serum as measured by the retention of strand-breaking activity. Col E1 plasmid DNA (30 μg/ml) was incubated with samples of free NCS and NCS conjugates which had been preincubated in human serum at a NCS-concentration of 1 μg/ml for the indicated time periods. The data clearly indicate that there is a rapid loss of NCS activity when incubated in the free form, whereas the conjugated NCS extends the activity of the NCS to a longer half-life. The data from a 10-minute incubation with NCS allows us to calculate a half-life of only 7 minutes for NCS, but the more relevant 1 hour incubation with DNA shows the effective half-life is somewhat greater. From these data an approximate value of an at least 3-fold increase in half-life from conjugation of NCS to antibody can be calculated.

Fig. 6. Serum stability of free and conjugated NCS measured by a DNA strand-break assay, using either a 10-minute or 60-minute DNA incubation period.

○, ● Free NCS, △, ▲ NCS-conjugate, ● and ▲ a 10-minute DNA incubation period, ○ and △ a 60-minute DNA incubation period.



Strand-breaks per molecule were plotted against preincubation time in human serum.

Discussion

It is important for effective therapeutic action that a drug should reach its target tissue in its active form. This is a particular problem for NCS which has both a short activity half-life and a poor retention and biodistribution. There is evidence that NCS conjugates are stabilized as a result of the substitution of one or both of the amino groups present in the molecule. This is probably not due to prevention of the apoprotein from proteolysis as suggested by MAEDA,[12] but simply the consequence of a hindered dissociation of NCS-chromophore from its protecting apoprotein. The chromophore is converted to an inactive species after leaving the apoprotein,[22] therefore only chromophore bound to and protected by apoprotein will reach its target in active form. It has already been shown that by conjugation to SMA the pharmacologic parameters can be improved, and the resulting lipophilic conjugate then localizes more readily in lymphoid tissue.[23] We have attempted to improve this targeting still further by the conjugation to a monoclonal antibody, which could in theory provide targeting to a number of different tumors depending on the antibody specificity. The work presented shows that such conjugation not only decreases the clearance of drug from the blood and its excretion from the body, but also that the activity half-life of the drug is improved. It has been shown further that these effects are accompanied by an improved tumor localization which shows specificity for antigen. This biodistribution remains to be described in terms of amount of active NCS per g of tumor tissue which can be localized within the time period of activity of NCS in serum.

Future work will be directed to measuring the toxicity and efficacy of antibody NCS conjugates to assess whether the improvements seen in biodistribution and serum stability will produce a more useful therapeutic agent.

Acknowledgment

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