Utilization of a Right-handed Coiled-coil Protein from Archaebacterium *Staphylothermus marinus* as a Carrier for Cisplatin

MATHILDA ERIKSSON[1], SAADIA HASSAN[2], ROLF LARSSON[2], STIG LINDER[1], TORBJÖRN RAMQVIST[1], HENRIK LÖVBORG[3], TRINE VIKINGE[3], EGBERT FIGGEMEIER[4], JÜRGEN MÜLLER[4], JÖRG STETEFELD[5], TINA DALIANIS[1*] and SUAT ÖZBEK[6*]

[1]*Karolinska Institutet, Department of Oncology-Pathology, Cancer Center Karolinska R8:01, Karolinska University Hospital, 171 76 Stockholm;*
[2]*Uppsala University, Department of Medical Sciences, Clinical Pharmacology, Uppsala University Hospital, 751 85 Uppsala;*
[3]*Optovent AB, SE-581 12 Linköping, Sweden;*
[4]*University of Basel, Institute of Inorganic Chemistry, Department of Chemistry, 4056 Basel, Switzerland;*
[5]*University of Manitoba, Department of Chemistry, Winnipeg R3T 2N2, Manitoba, Canada;*
[6]*Heidelberger Institut für Zoologie, Abteilung Molekulare Evolution und Genomik, 69120 Heidelberg, Germany*

Abstract. *Background: The nano-sized right-handed coiled-coil (RHCC) protein, originating from the archaebacterium Staphylothermus marinus, is stable at high salt concentrations, high temperatures, high pressures and extremes of pH. Its crystal structure reveals four hydrophobic cavities which can incorporate heavy metals. Nano-sized compounds have been used to carry cytotoxic drugs to tumours, avoiding delivery to healthy tissue, in part due to enhanced permeability in tumour blood vessels (enhanced permeability and retention effect). Materials and Methods: The ability of RHCC to carry the platinum-containing chemotherapeutic drug cisplatin to cells, while retaining the cytotoxic potential was tested both in vitro and in vivo. Results: RHCC was able to bind and enter cells in vitro and was not severely toxic or immunogenic in mice. Moreover, RHCC incorporated cisplatin, without inhibiting the cytotoxic potential of the drug against tumour cell lines in vitro or in vivo. Conclusion: RHCC can be used as a carrier of cisplatin without abrogating the effect of the drug.*

*Both authors contributed equally to this work.

Correspondence to: Tina Dalianis, Karolinska Institutet, Department of Oncology-Pathology, Cancer Center Karolinska R8:01, Karolinska University Hospital, 171 76 Stockholm, Sweden. Tel: +46 8517 76583, Fax: +46 851776630, e-mail: Tina.Dalianis@ki.se

Key Words: RHCC, cisplatin, nano, carrier.

The right-handed coiled-coil protein, RHCC, is part of the tetrabrachion complex that constitutes the surface layer of the cell envelope of the archaebacterium *Staphylothermus marinus*. RHCC is composed of an α-helical domain, made up of four strands oriented in parallel in a right-handed fashion (1-3). RHCC has been shown to be stable at high salt concentrations, temperatures of over 100˚C, high pressures and extreme ranges of pH (1). Such characteristics are probably due to the adjustment of the bacteria to their environment in heated places on the ocean floor (4). The crystal structure of the protein shows an axial channel with four hydrophobic cavities (3), naturally occupied by water molecules, but capable of incorporating heavy metals (Figure 1). Screenings of cationic, anionic and multi-ionic heterocomplexes of mercury, platinum, osmium, and uranium have previously been investigated to determine phase information for RHCC crystals (3, 5). The RHCC tetramer has a molecular weight of 22.8 kDa and an average length and diameter of 72 Å and 25 Å, respectively (1, 3).

The platinum-containing chemotherapeutic drug *cis*-diammine-dichloroplatinum (II) (cisplatin) (6) is one of the most potent and curative antitumour drugs available: it is very effective in the treatment of testicular carcinoma and it is also used for ovarian, cervical, head and neck, non-small cell lung cancer, bladder and stomach cancers (7). In fact, more than half of all cancer patients treated using chemotherapy receive platinum complexes (8). However, cisplatin causes a number of adverse effects,

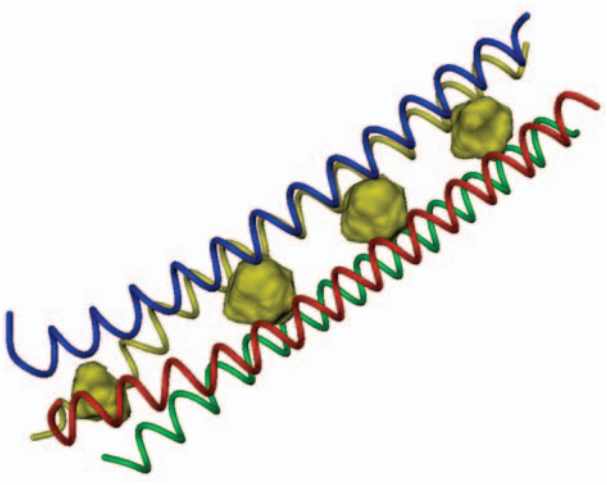

Figure 1. *Structural model of the RHCC protein. Side view of the tetrameric channel, where the backbone is shown in ribbon representation with different colours for each helical chain, and the four large cavities are drawn in yellow.*

including serious and dose-limiting nephrotoxicity, ototoxicity and neurotoxicity, of which the latter two are usually irreversible (9-11).

The ultimate goal of this work is to explore the potential of cisplatin incorporated into RHCC (RHCC/C) to reduce the negative adverse effects of cisplatin treatment by giving a more precise delivery of the drug to cancer cells, thereby avoiding delivery to normal cells. This hypothesis is based on the "enhanced permeability and retention (EPR) effect", which states that tumour vessels are leaky and allow macromolecular extravasation and that tumours lack effective lymphatic drainage preventing clearance of macromolecules and promoting their accumulation in the tumour (12). It has previously been shown that the conjugation of low-molecular-weight drugs, such as cisplatin (298 Da), to polymeric carriers passively targets the chemotherapeutic agent to solid tumours in both animal models and man (13). The increased size of the drug complex might also reduce cisplatin induced ototoxicity, since larger molecules are more unlikely to penetrate the blood-perilymph barrier separating the inner ear from the systemic circulation, a system similar to the blood-brain barrier (14, 15).

In this study, representing the initial investigations towards our ultimate goal, the ability of RHCC to incorporate cisplatin, and bind to and enter cells *in vitro* was first investigated. Thereafter, conventional cisplatin treatment was compared to RHCC/C treatment regarding the ability to kill tumour cells *in vitro* and in a pilot experiment also *in vivo*. In parallel, RHCC was delivered *in vivo* to mice, to examine to what extent it induced an immune response and if it influenced the viability of the mice.

Materials and Methods

Production and purification of RHCC. Recombinant RHCC polypeptide chain fragments were produced in *Escherichia coli* (3). A synthetic gene encoding residues Ile 3-Ile 52 of the tetrabrachion sequence was ligated into the BamH/EcoRI site of pet15b, and expressed in *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany). The RHCC was purified from the bacterial lysates by nickel-nitrilotriacetic acid (Ni-NTA) sepharose affinity chromatography (Qiagen, Hilden, Germany) under denaturing conditions and refolded in physiological buffer conditions on the Ni-NTA column. The polyhistidine tag was cleaved using thrombin and the cleaved peptide removed by Ni-NTA. The RHCC solution was purified from bacterial endotoxins by incubation with Polymyxin B-agarose (Sigma-Aldrich, Stockholm, Sweden) at 4˚C for 1 h with rotation. The protein concentration was measured using NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). The purified RHCC ranged from 10-15 mg/l culture.

Incorporation of cisplatin into RHCC. The RHCC and cisplatin in aqueous solution (Mayne, Warwickshire, UK) were mixed using 1 mg of each at room temperature (RT) for 1 h and then centrifuged at 14 krpm for 5 min to remove undissolved cisplatin. The supernatant was run on a PD-10 desalting column (containing Sephadex G-25 medium) according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden) to remove unbound cisplatin. The protein concentration was measured by NanoDrop and the platinum concentrations were measured by inductively-coupled plasma optical emission spectrometry (ICP-OES) at 214.424 nm. The RHCC with incorporated cisplatin was denominated RHCC/C.

Conjugation of RHCC with Alexa Fluor 488 5-sulfodichlorophenol ester. The RHCC was conjugated to Alexa Fluor 488 5-sulfodichlorophenol ester (SDP) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RHCC was mixed with Alexa Fluor 488 SDP reactive dye for 1 h and the conjugated protein was separated from free dye on a PD-10 desalting column. The Alexa Fluor 488 SDP:protein ratio was ~1.5 moles of dye per mole protein as measured by NanoDrop. RHCC conjugated to Alexa Fluor 488 SDP was denominated AF-RHCC.

Flow cytometry. A total of 5×10^4 FaDu cells (head and neck squamous cell carcinoma) (16) in 300 μl cell culture medium were incubated with 0.1-100 μg of AF-RHCC for 10 min - 8 h, at 4˚C or 37˚C, washed twice in PBS and analyzed by flow cytometry in a FACS Calibur using Cell Quest software (Becton Dickinson, San Jose, CA, USA).

Fluorescence and confocal laser scanning microscopy. A total of 5×10^4 FaDu cells/well seeded in 300 μl FaDu-medium overnight (o.n.) at 37˚C in 8-chamber microscopy slides (BD Biosciences, San Diego, CA, USA) were mixed with 100 μg AF-RHCC for 10 min - 8 h, at 4˚C or 37˚C. The cells were washed three times in PBS, fixed in 3% paraformaldehyde (Sigma-Aldrich) for 15 min, washed three times, and mounted with Vectashield HardSet medium with 4',6-diamidino-2-phenylindole (DAPI) (Immunkemi, Järfälla, Sweden). The cells were photographed at ×40 magnification using a Zeiss Axioplan 2 microscope with Zeiss AxioVision software (Carl Zeiss AB, Stockholm, Sweden), and ×60 magnification in a Nikon Eclipse

TE 300 confocal laser scanning microscope (Carl Zeiss AB) with Ultra View software (Perkin Elmer, Waltham, MA, USA).

Human cell lines and primary human tumour cells (PHTC). Cisplatin-sensitive and -resistant cell lines were tested, as well as cells originating from tumour types currently treated with cisplatin. The FaDu cells were grown in Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated foetal bovine serum (FBS), 0.1 mM MEM non-essential amino acids, 1.2 mM sodium pyruvate, 24 mM HEPES, with penicillin and streptomycin. MDA 231 a breast cancer cell line, RPMI 8226/S a myeloma cell line and its resistant sub-line 8226/Dox40, NCI-H69 a small lung cancer cell line and its resistant sub-line H69AR, ACHN a primary resistant adenocarcinoma, and the ovarian carcinoma cell line A2780 and its resistant sub-line A2780-Cis, were all grown in RPMI-1640 with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, penicillin and streptomycin. hTERT-RPE1 a normal epithelial telomerase immortalized line was grown in DMEM nutrient mixture F-12 Ham with 10% heat-inactivated FCS, 2 mM glutamine, penicillin and streptomycin. Tumour cells obtained from three ovarian carcinoma patients after ethical approval (Uppsala University Ethical Committee) were isolated by collagenase dispersion and Percoll density gradient centrifugation (GE Healthcare Life Sciences, Uppsala, Sweden). The cells (minimum 70% viability) were frozen in FCS with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for 24 h at –70˚C and stored at –150˚C, which does not affect drug sensitivity (17). For the experiments, the frozen cells were thawed, washed twice and kept in RPMI-1640.

Fluorometric microculture cytotoxicity assay (FMCA). RHCC, RHCC/C and cisplatin were tested in triplicate at six concentrations by two-fold serial dilution in 0.1 M NaCl, starting at 3.48 μM for RHCC and RHCC/C (with 10 μM cisplatin) and 10 μM for cisplatin. Microtitre plates (Nunc, Roskilde, Denmark) were prepared with 20 μl/well of drug solution at ten times the desired concentration. To evaluate the cytotoxic activity, the drug plates were seeded with 2×10^4 cells/180 μl/well. A column without drugs served as control and a column with medium alone served as blank. FMCA is based on measurements of fluorescence generated from the hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes (17). The plates were incubated at 37˚C for 72 h, washed in PBS, whereafter FDA (dissolved in PBS, 10 μg/ml) was added at 100 μl/well. The plates were then incubated for 50 min and fluorescence/well was measured at 538 nm in a scanning fluorometer (Fluoroscan II, Labsystems Oy, Helsinki, Finland). Fluorescence is proportional to the number of viable cells/well. Quality criteria for successful analysis included a fluorescence signal in control wells of more than five times the mean blank value and a mean coefficient of variation in control wells of less than 30% . The experiments were repeated three times.

Animals. Balb/c mice in open cages and severe combined immunodeficient (SCID) mice in individually ventilated cages (IVC) were bred and kept at the Microbiology and Tumour Biology Center, Karolinska Institutet.

In vivo tumour reduction assay. The SCID mice were injected subcutaneously (*s.c.*) with 5×10^5 FaDu cells in 100 μl PBS, and one week later injected intravenously (*i.v.*) with 1.0 mg/kg cisplatin, 0.675 mg RHCC/C with ~0.35 mg/kg cisplatin, or 0.1 M NaCl (5 mice/group). In a separate experiment, after tumour challenge, 5 mice were treated with 0.675 mg RHCC. The mice were palpated and weighed three times/week, and euthanized if the tumour diameter exceeded 10 mm or if weight decreased below 80% of starting weight, according to the ethical guidelines.

Enzyme-linked immunosorbent spot (EliSpot) assay. IFN-γ EliSpot assays were performed according to the manufacturer's instructions (Mabtech, Nacka, Sweden). Splenocytes (1.2×10^5) from Balb/c mice collected 7 days after *i.v.* injection of 0.4 or 0.2 mg RHCC (two mice/group), or 0.1 M NaCl (one control mouse) were cultured in triplicate for 40 h in anti-mouse IFN-γ antibody coated EliSpot plates (Millipore AB, Solna, Sweden) alone, or with 1, 5, or 10 μg/ml of RHCC, or a lymphocytic choriomeningitis virus (LCMV)-derived nucleoprotein peptide ($NP_{118-126}$) (RPQASGVYM) (18) as negative control, or phorbol myristate acetate (PMA) (25 ng/ml) and ionomycin (IO) (250 ng/ml) (Sigma-Aldrich) as positive control. The spots were counted in an EliSpot reader and processed by EliSpot Reader 4.0 software (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Enzyme-linked immunosorbent assay (ELISA). The antibody response to RHCC in the sera from Balb/c mice collected 14 days after *i.v.* injection of 0.4 or 0.2 mg RHCC (two mice/group), or 0.1 M NaCl (one mouse), was measured by ELISA. Microtitre plates (BD Biosciences) were coated with 5 μg/ml RHCC in 0.1 M carbonate-buffer (pH 9.6) per well o.n. at 4˚C. The plates were blocked in blocking solution (5% milk powder, 0.2% Tween in PBS) for 1 h at RT. Serial dilutions of the mouse sera in blocking solution (1:50-1:1,350) were added in duplicates to the wells. After 1 h at RT, the plates were washed and incubated with secondary alkaline phosphatase (AP) conjugated goat-anti mouse IgG antibody (Sigma-Aldrich). The plates were washed and developed with nitrophenylphosphate (pNPP) (Sigma-Aldrich) and the absorbance was measured at 405 nm in a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Dendritic cell maturation assay. Murine bone marrow-derived dendritic cells (BMDCs) were generated according to Lutz *et al.* (19). The DCs were seeded in 24-well plates (BD Biosciences) at 10^6 cells/ml in R10 medium (19) and mixed with 50 μg/ml RHCC. Lipopolysaccharide (LPS) 1 μg/ml (Sigma-Aldrich) was used as positive control. After 24 h stimulation, the DCs were stained with phycoerythrin-conjugated (PE) antibodies to CD40, CD80 and CD86 and fluorescein isothiocyanate-conjugated (FITC) antibody to major histocompatibility complex (MHC) Class II $I\text{-}A^b$ (BD Biosciences) for 30 min at 4˚C, washed twice in PBS with 0.1% bovine serum albumin (BSA) and analyzed by flow cytometry. Interleukin-12 (IL-12) production was analyzed by ELISA according to the manufacturer's instructions (Mabtech). The plates were coated with 2 μg/ml of anti-IL-12 capture antibody o.n., then after blocking, 100 μl of BMDC culture supernatants were added at dilutions 1:1-1:128 for 2 h at RT. The plates were thereafter incubated with biotinylated anti-IL-12 antibody 1 h at RT and the response was visualized as above for ELISA.

Statistical analyses. The FMCA data was processed by GraphPad Prism (GraphPad Software, Inc. San Diego, CA, USA) with non-linear regression to a standard sigmoidal dose-response model. Zero and 100% cell survival were set as the maximum effect and the baseline and IC_{50} (inhibitory concentration 50%) was estimated.

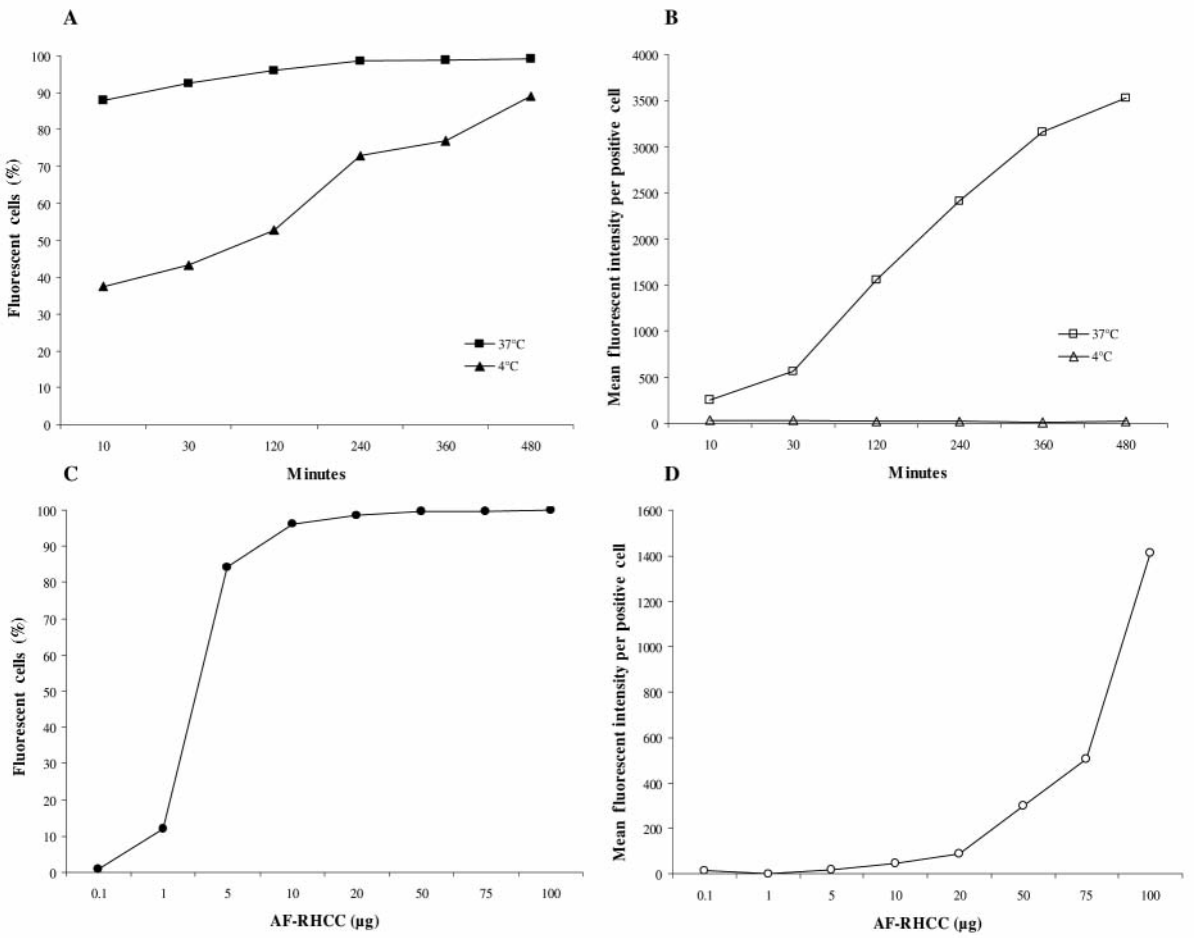

Figure 2. *Kinetics and dose-response of AF-RHCC-binding to FaDu cells. A-B, Binding after 10 min – 8 h, at 4˚C or 37˚C. C-D, Binding after incubation with 0.1-100 µg, at 37˚C.*

For drugs not resulting in 50% reduction of cell survival the IC_{50} was set as being greater than the highest concentration tested.

Results

Incorporation of cisplatin into RHCC. The molar ratio between cisplatin and the RHCC tetramer ranged from 0.9-1.0, indicating that on average, one cavity in each coiled-coil tetramer was occupied by cisplatin. The RHCC/C complex was stable in solution up to 12 h, shown by repeated measurements during dialysis against cisplatin-free buffer. However, the molar ratio declined to 0.5 after 24 h, probably due to slow diffusion from the coiled-coil cavities.

RHCC binding and uptake in FaDu cells. AF-RHCC bound better to the FaDu cells at 37˚C compared to 4˚C. After 10 min at 37˚C, 90% of the cells already exhibited significant binding of AF-RHCC and most of the cells (99%) exhibited fluorescence after 8 h incubation (Figure 2A). In contrast, at 4˚C, binding was slower and with less AF-RHCC bound/cell (Figure 2B). After incubation of 10 µg AF-RHCC with 5×10^4 FaDu cells, most of the cells (96%) displayed binding of the protein, and with 50 µg, practically all the cells (99.6%) bound AF-RHCC (Figure 2C). However, the amount of AF-RHCC bound to each cell continued to rise and saturation was still not detected following incubation with 100 µg AF-RHCC (Figure 2D). Incubation of the FaDu cells with AF-RHCC at 37˚C for 10-60 min resulted in a barely detectable fluorescent signal with conventional fluorescence microscopy (Figures 3A and B), while 4-8 h of incubation gave a spotty pattern, suggesting the AF-RHCC was inside the cells (Figures 3C and D). By confocal laser scanning microscopy, the staining was verified to be in the cytoplasm of the cells, and the staining was spotty, indicating uptake of the protein into intracellular vesicles (Figure 3E). Almost no fluorescence was seen bound to or associated with cells incubated with AF-RHCC at 4˚C (data not shown).

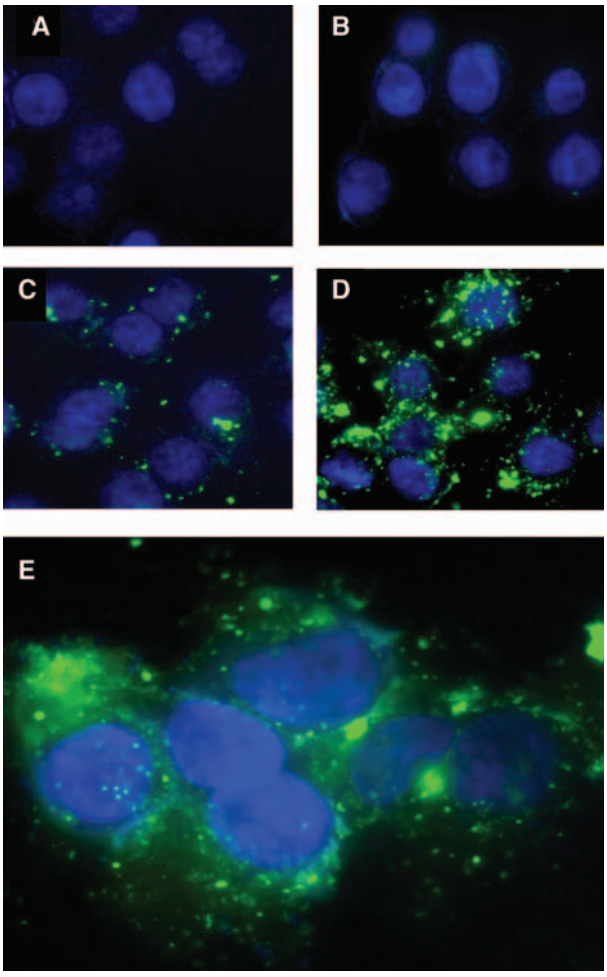

Figure 3. *Kinetics of AF-RHCC uptake and cellular localization in FaDu cells. Fluorescent microscopy images (×40 magnification) after incubation for 10 min (A), 60 min (B), 4 h (C) or 8 h (D), at 37˚C. E, Confocal laser scanning microscopy image (×60 magnification) after incubation for 8 h at 37˚C. Blue colour: DAPI-staining of DNA in the nucleus, green colour: AF-RHCC.*

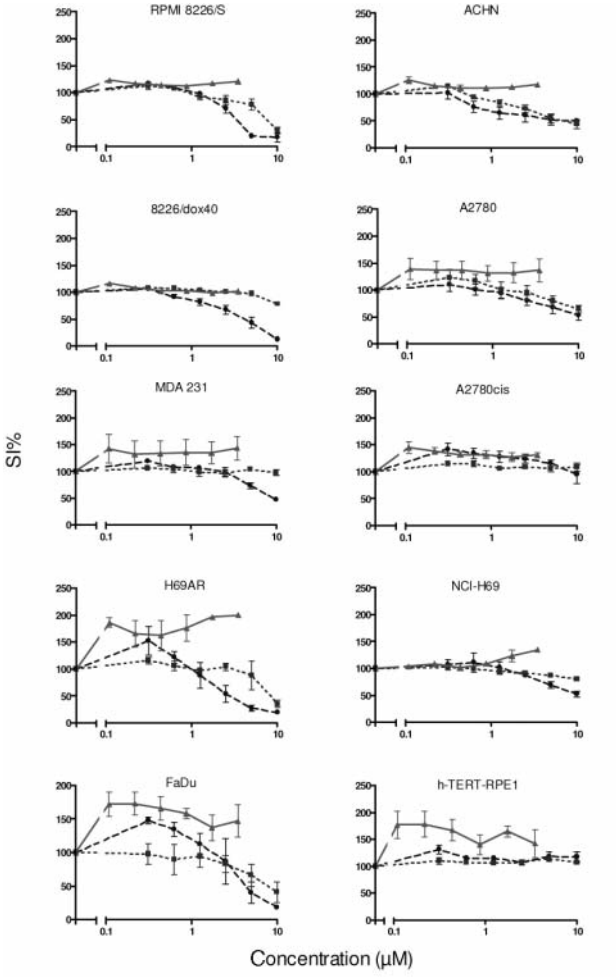

Figure 4. *The in vitro cytotoxic effect in ten tumour cell lines of (▲) RHCC, (■) cisplatin and (●) RHCC/C. SI% : Survival index, defined as the fluorescence in experimental wells as a percentage of that in control wells, with blank values subtracted. Results are presented as mean ± SEM of three independent experiments.*

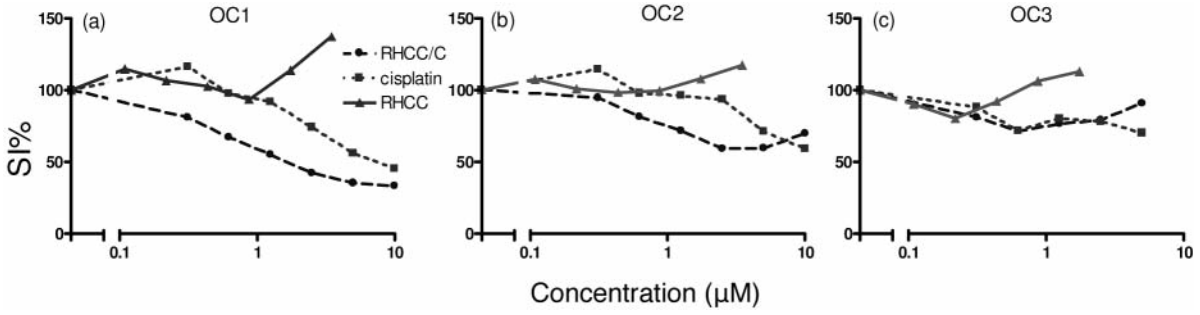

Figure 5. *The in vitro cytotoxic effect in primary human tumour cells from three patients (OC1-3) diagnosed with ovarian cancer of (▲) RHCC, (■) cisplatin and (●) RHCC/C. SI% : Survival index, defined as the fluorescence in experimental wells as a percentage of that in control wells, with blank values subtracted. Results are presented as mean ± SEM of three independent experiments.*

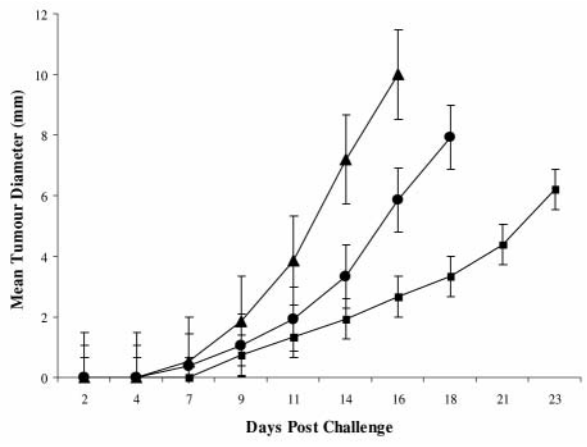

Figure 6. *In vivo tumour reduction from RHCC/C and cisplatin. Mean tumour size in mice treated with (■) cisplatin, (●) RHCC/C, or (▲) NaCl. Results are presented as mean ± SEM.*

Table I. *Estimated IC_{50} (log IC_{50}± SEM) of RHCC/C and cisplatin in ten cell lines. Where RHCC/C or cisplatin treatment did not result in 50% reduction of cell survival at the highest concentration tested (10 μM), the IC_{50} was set to >10 μM.*

Cell line	RHCC/C IC_{50} (μM) (95% confidence interval)	Cisplatin IC_{50} (μM) (95% confidence interval)
RPMI 8226/S	3.37 (2.7-4.1)	7.44 (6.0-9.1)
8226/dox40	3.82 (3.1-4.7)	>10
MDA 231	9.16 (7.3-11.5)	>10
H69AR	3.08 (1.8-5.4)	8.50 (6.7-10.8)
FaDu	4.66 (2.6-8.3)	7.90 (4.2-14.8)
ACHN	6.49 (2.8-15.3)	6.90 (4.8-9.9)
A2780	>10	>10
A2780cis	>10	>10
NCI-H69	>10	>10
h-TERT-RPE1	>10	>10

Cytotoxicity in vitro. A dose-dependent decrease of viability of the tumour cells was observed for RHCC/C and cisplatin in most of the evaluated cell lines. Figure 4 displays the concentration-effect curves in all the cell lines studied and Table I shows the corresponding IC_{50} values. RHCC was nontoxic at the tested concentrations and generally RHCC/C and cisplatin had a similar effect in all the tested cell types. However, RHCC/C showed a significantly higher effect than cisplatin in the myeloma cell line RPMI 8226/S ($p<0.001$), its sub-line 8226/dox40 (where IC_{50} for cisplatin could not be estimated), the adenocarcinoma breast cancer cell line MDA 231 (where IC_{50} for cisplatin could not be estimated), and the small-cell lung cancer sub-line H69AR ($p<0.0015$). The RHCC/C tended to be slightly more efficient than cisplatin in the FaDu cell line, but both agents displayed similar activity in the renal adenocarcinoma primary resistant cell line ACHN, the ovarian carcinoma cell lines A2780 and A2780cis and the small-cell lung cancer cell line NCI H69 while the IC_{50} was not reached for some of these. The normal epithelial telomerase immortalized hTERT-RPE1 cell line was not sensitive to RHCC/C or cisplatin. Figure 5 illustrates the concentration-response curves in the tumour cell samples from the three patients with ovarian carcinoma (OC1-3). RHCC/C showed higher activity in samples OC1 and OC2 compared to cisplatin (Figures 5A and B), while both had a similar effect on the tumour cells from the third patient (Figure 5C).

Effect of RHCC/C in vivo. The cytotoxic effect of cisplatin was retained after coupling to RHCC (Figure 6) and there was no significant alteration in weight observed in any animal during the experiment (data not shown). In a separate experiment, where mice were treated with RHCC alone, it could be seen that RHCC itself had no effect on tumour reduction (data not shown).

Humoral and cellular immune response to RHCC. RHCC induced a slight production of specific $CD8^+$ T-cells as seen by IFNγ-EliSpot (while PMA/IO stimulation induced unspecific IFNγ-secretion from $700\text{-}800\times10^6$ cells, data not shown) (Figure 7A), but no significant antibody response in the ELISA (Figure 7B). RHCC induced a very marginal maturation of DCs as seen by flow cytometry and ELISA experiments, with only a weak increase in CD40 expression and IL-12 production and no increase in the other tested maturation markers CD80, CD86 and MHC Class II, as compared to that in the unstimulated cells (Figures 7C and D). However, it is important to note that non-endotoxin purified RHCC induced a high unspecific reactivity in all immune response assays (data not shown). Mice showed no signs of illness with regard to viability or alterations in weight after injection with endotoxin purified RHCC.

Discussion

RHCC was shown to stably incorporate cisplatin over a range of up to 12 h at RT which confirmed that the complex was stable enough in solution to be applied *in vivo* where RHCC/C could function as a nano-carrier.

RHCC was also shown to bind to and enter the cytoplasm of FaDu cells at 37˚C. The RHCC bound to almost 100% of the cells after a very short incubation time *in vitro*, which at first seemed alarming, indicating that the protein would not have enough time to reach the tumour when injected *in vivo*, however, it became clear that a high proportion of free protein remained outside the cells after several hours of

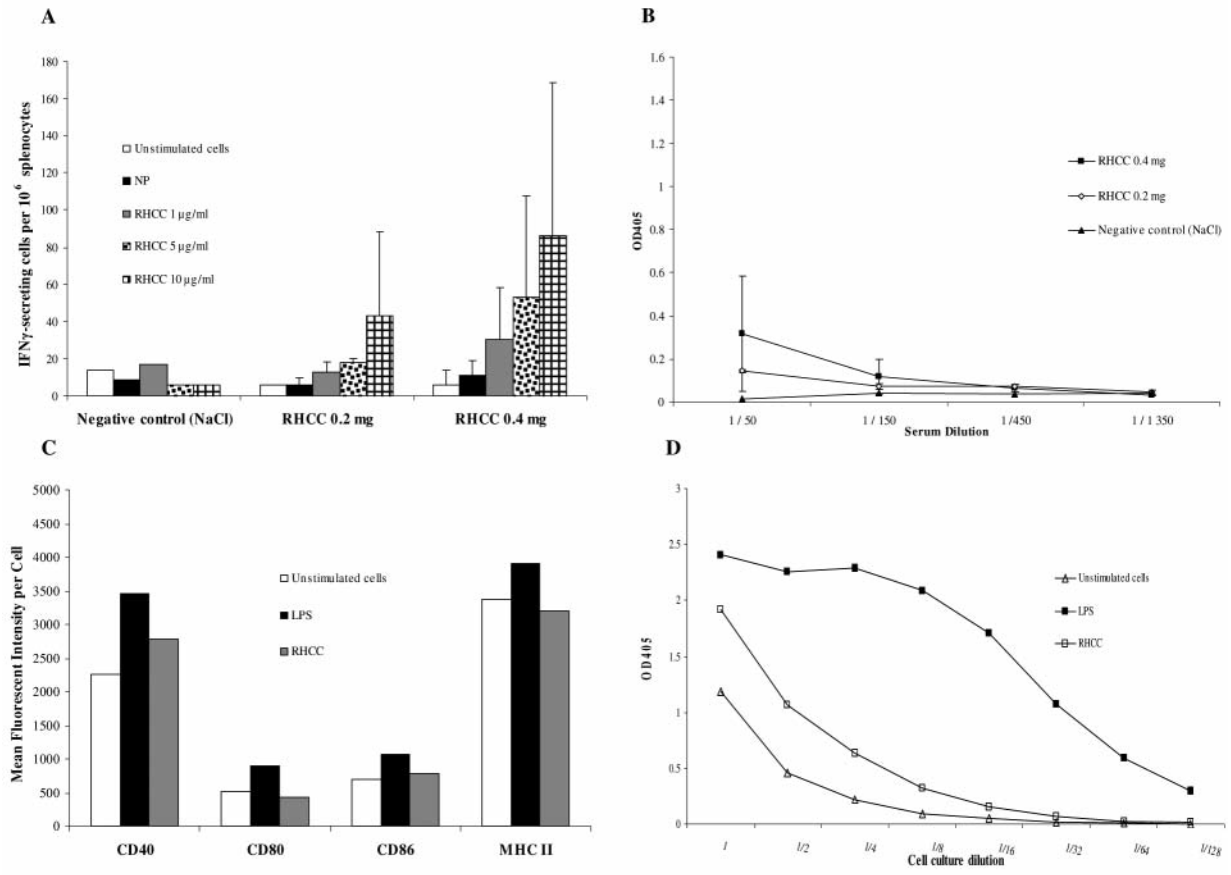

Figure 7. *Immune response, in mice after RHCC injection, and in DCs after co-culture. A, The number of IFNγ-secreting cells per 10^6 murine splenocytes (stimulation with PMA/IO resulted in IFNγ-secretion from 700-800×10^6 cells). B, RHCC-specific antibody titres in murine serum. C, The mean fluorescence of four different antibodies specific for surface maturation markers CD40, CD80, CD86 and MHC class II in murine DCs. D, IL-12 levels in serial diluted cell supernatants from murine DCs. Results are presented as mean ± SEM. NP: LCMV-derived nucleoprotein peptide, LPS: lipopolysaccharide.*

incubation. These data thus demonstrated that the protein bound to the cells very efficiently, but that the uptake was potentially not too fast for *in vivo* administration.

The fact that the binding of RHCC to cells was so much more efficient at 37˚C compared to 4˚C, as seen in the flow cytometric experiments, could indicate an unidentified active energy-dependent mechanism for cellular uptake. As seen by microscopy, the uptake of RHCC into the cells at 4˚C was almost non-existent, while at 37˚C much of the protein could be seen inside the cell cytoplasm. The spotty pattern could indicate an uptake in a specific subcellular compartment, such as the lysosome. Further studies to elucidate the exact subcellular location of RHCC are warranted.

RHCC/C, but not RHCC, also induced cytotoxicity *in vitro* as shown by the FMCA, a method that has been extensively used for determining drug activity on human tumour cell lines as well as on primary tumour cells from patients with leukaemia and solid tumour malignancies. Moreover, results of different drugs tested with the FMCA on primary human tumour cultures have correlated very well with the clinical activity profile of that particular drug (17). In the current study, RHCC was not toxic at the tested concentration in any of the studied cell types. Moreover, RHCC/C and cisplatin, when used at equimolar cisplatin concentrations, induced similar cytotoxic effects against the different tumour types. In fact, for many cell lines, as well as for primary human tumour cells, RHCC/C was even more active than cisplatin. It was remarkable also that RHCC/C was more effective than cisplatin in two drug-resistant cell lines: the myeloma 8226/dox40 and the small-cell lung cancer H69AR, possibly suggesting a different mode of entry or action. As mentioned above, RHCC alone had no cytotoxic effect in any of the cell lines and patient samples tested, or in the *in vivo* mouse tumour model, but its potentiating effect on cisplatin activity suggested that it may have a positive influence on the capability of cisplatin to penetrate the tumour cells. Furthermore, these *in vitro* results indicated that coupling of cisplatin to RHCC did not attenuate the cytotoxic effect of cisplatin.

In the single *in vivo* pilot experiment RHCC/C conferred antitumor activity. However, for ethical reasons, using the present RHCC:cisplatin ratios it was not possible to inject sufficient RHCC/C to equal the concentration of cisplatin alone. Therefore a direct comparison of RHCC/C *versus* cisplatin was not possible. The magnitude of the antitumor effect that was shown by the relatively low cisplatin dose was however promising. Further experiments designed in a different way to solve these issues are underway.

RHCC, purified from endotoxins, showed no obvious toxicity and very slight immunoreactivity, since it caused a very slight specific $CD8^+$ T-cell activity, marginal DC maturation and induced almost no specific antibodies. While these results were very encouraging it should, however, be mentioned that the RHCC preparation must be purified extensively from endotoxins in order not to obtain a broad unspecific immune response (unpublished results).

In summary, for the first time, it was shown that RHCC can bind to and enter into cells and when purified from endotoxins it does not induce a major immune response *in vivo*. Moreover, RHCC can incorporate cisplatin and retain, and even enhance, the cytotoxic potential of the drug against a variety of tumour cell lines and primary explanted tumours *in vitro*, and preliminary *in vivo* data look promising. It was thereby shown that RHCC can be used as a novel carrier of cisplatin without abrogating the effect of the drug. It would now be worthwhile pursuing further *in vivo* analyses, such as biodistribution studies of RHCC/C, tumour reduction assays with different doses of RHCC/C and cisplatin, as well as to test if RHCC/C also reduces the toxic potential of cisplatin *in vivo*.

Acknowledgements

We thank Maria Hägg for providing us with the FaDu cell line. Optovent AB, the Swedish Cancer Foundations, the Swedish Research Council, the Stockholm Cancer Society, the Karolinska Institutet and the Swedish City Council are all acknowledged for financial support.

References

1 Peters J, Nitsch M, Kuhlmorgen B *et al*: Tetrabrachion: a filamentous archaebacterial surface protein assembly of unusual structure and extreme stability. J Mol Biol *245(4)*: 385-401, 1995.

2 Peters J, Baumeister W and Lupas A: Hyperthermostable surface layer protein tetrabrachion from the archaebacterium *Staphylothermus marinus*: evidence for the presence of a right-handed coiled coil derived from the primary structure. J Mol Biol *257(5)*: 1031-1041, 1996.

3 Stetefeld J, Jenny M, Schulthess T, Landwehr R, Engel J and Kammerer RA: Crystal structure of a naturally occurring parallel right-handed coiled coil tetramer. Nat Struct Biol *7(9)*: 772-776, 2000.

4 Fiala G, Stetter KO, H.W J, Langworthy TA and Madon J: *Staphylothermus marinus* sp. nov. represents a novel genus of extreme thermophilic submarine heterotrophic archaebacteria growing up to 98˚C. System Appl Microbiol *8*: 106-113, 1986.

5 Ozbek S, Muller JF, Figgemeier E and Stetefeld J: Favourable mediation of crystal contacts by cocoamidopropylbetaine (CAPB). Acta Crystallogr D Biol Crystallogr *61(Pt 4)*: 477-480, 2005.

6 Rosenberg B, Van Camp L and Krigas T: Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature *205*: 698-699, 1965.

7 Boulikas T and Vougiouka M: Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs (review). Oncol Rep *11(3)*: 559-595, 2004.

8 Siafaca K: Oncology trends products markets – Part 1. Future Oncology pp. 1045-1071, 1999.

9 Piel IJ, Meyer D, Perlia CP and Wolfe VI: Effects of *cis*-diamminedichloroplatinum (NSC-119875) on hearing function in man. Cancer Chemother Rep *58(6)*: 871-875, 1974.

10 Alberts DS and Noel JK: Cisplatin-associated neurotoxicity: can it be prevented? Anticancer Drugs *6(3)*: 369-383, 1995.

11 Quasthoff S and Hartung HP: Chemotherapy-induced peripheral neuropathy. J Neurol *249(1)*: 9-17, 2002.

12 Maeda H and Matsumura Y: Tumoritropic and lymphotropic principles of macromolecular drugs. Crit Rev Ther Drug Carrier Syst *6(3)*: 193-210, 1989.

13 Muggia FM: Doxorubicin-polymer conjugates: further demonstration of the concept of enhanced permeability and retention. Clin Cancer Res *5(1)*: 7-8, 1999.

14 Jahnke K: The blood-perilymph barrier. Arch Otorhinolaryngol *228(1)*: 29-34, 1980.

15 Juhn SK, Rybak LP and Fowlks WL: Transport characteristics of the blood-perilymph barrier. Am J Otolaryngol *3(6)*: 392-396, 1982.

16 Rangan SR: A new human cell line (FaDu) from a hypopharyngeal carcinoma. Cancer *29(1)*: 117-121, 1972.

17 Lindhagen E, Nygren P and Larsson R: The fluorometric microculture cytotoxicity assay. Nat Protoc *3(8)*: 1364-1369, 2008.

18 Shen H, Miller JF, Fan X, Kolwyck D, Ahmed R and Harty JT: Compartmentalization of bacterial antigens: differential effects on priming of CD8 T-cells and protective immunity. Cell *92(4)*: 535-545, 1998.

19 Lutz MB, Kukutsch N, Ogilvie AL *et al*: An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods *223(1)*: 77-92, 1999.

Received July 7, 2008
Revised October 6, 2008
Accepted November 6, 2008