Articles

Novel Peptide Conjugates for Tumor-Specific Chemotherapy[§]

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One of the major problems in cancer chemotherapy are the severe side effects that limit the dose of the anticancer drugs because of their unselectivity for tumor versus normal cells. In the present work, we show that coupling of anthracyclines to peptides is a promising approach to obtain selectivity. The peptide−drug conjugate was designed to bind to specific receptors expressed on the tumor cells with subsequent internalization of the ligand−receptor complex. Neuropeptide Y (NPY), a 36-amino acid peptide of the pancreatic polypeptide family, was chosen as model peptide because NPY receptors are overexpressed in a number of neuroblastoma tumors and the thereof derived cell lines. Daunorubicin and doxorubicin, two widely used antineoplastic agents in tumor therapy, were covalently linked to NPY via two spacers that differ in stability: an acid-sensitive hydrazone bond at the 13-keto position of daunorubicin and a stable amide bond at the 3′-amino position of daunorubicin and doxorubicin. Receptor binding of these three conjugates ([C^{15}]-NPY-Dauno-HYD, [C^{15}]-NPY-Dauno-MBS, and [C^{15}]-NPY-Doxo-MBS) was determined at the human neuroblastoma cell line SK-N-MC, which selectively expresses the NPY Y_1 receptor subtype, and cytotoxic activity was evaluated using a XTT-based colorimetric cellular cytotoxicity assay. The different conjugates were able to bind to the receptor with affinities ranging from 25 to 51 nM, but only the compound containing the acid-sensitive bond ([C^{15}]-NPY-Dauno-HYD) showed cytotoxic activity comparable to the free daunorubicin. This cytotoxicity is Y_1 receptor-mediated as shown in blocking studies with BIBP 3226, because tumor cells that do not express NPY receptors were sensitive to free daunorubicin, but not to the peptide−drug conjugate. The intracellular distribution was investigated by confocal laser scanning microscopy. We found evidence that the active conjugate [C^{15}]-NPY-Dauno-HYD releases daunorubicin, which is localized close to the nucleus, whereas the inactive conjugate [C^{15}]-NPY-Dauno-MBS is distributed distantly from the nucleus and does not seem to release the drug within the cell.

Introduction

The anthracyclines daunorubicin (Dauno) and doxorubicin (Doxo) are widely used antineoplastic agents in the treatment of various types of cancer.[1,2] However, the clinical application of these drugs is limited by their toxic dose-related side effects: e.g. myelosuppression, nephrotoxicity, cardiotoxicity, and extravasation derived from the cytotoxicity toward all dividing cells.[3,4] To increase their therapeutic index, drug delivery systems are needed which transport the antineoplastic agents to the tumor (drug targeting). Various carriers have been investigated so far with research focusing mainly on macromolecular carriers such as monoclonal antibodies, liposomes, serum proteins, polyethylene glycol, and hydroxypropylmethacrylamide polymers.[5−12] Only recently, some promising approaches using peptides as vectors were investigated: e.g. pegelin and penetratin were used for transporting doxorubicin through the blood−brain barrier, and peptides that specifically target tumor blood vessels were identified by phage display and coupled to anticancer drugs.[13−15]

In this study, we investigate the usefulness of peptides as carriers for which the receptors are overexpressed on tumor cells. Covalent linking of the drug to the peptide could be used for chemotherapy and would lead to selective addressing of the tumor cells. As a model peptide, we used neuropeptide Y (NPY) because its receptors are produced in a number of neuroblastoma and the thereof derived cell lines. NPY is a 36-amino acid peptide of the pancreatic polypeptide family. It is expressed in the peripheral and central nervous systems and is one of the most abundant neuropeptides in the brain.[16] Five distinct NPY receptors have been cloned, which have been named Y_1, Y_2, Y_4, Y_5, and y_6 receptor subtypes.[17] Upon binding to the G-protein coupled receptors, the ligand−receptor complex is internalized, which provides a convenient way to enter the cell by

[§] Dedicated to Prof. Dr. Horst Kunz, University of Mainz, on the occasion of his 60th birthday.

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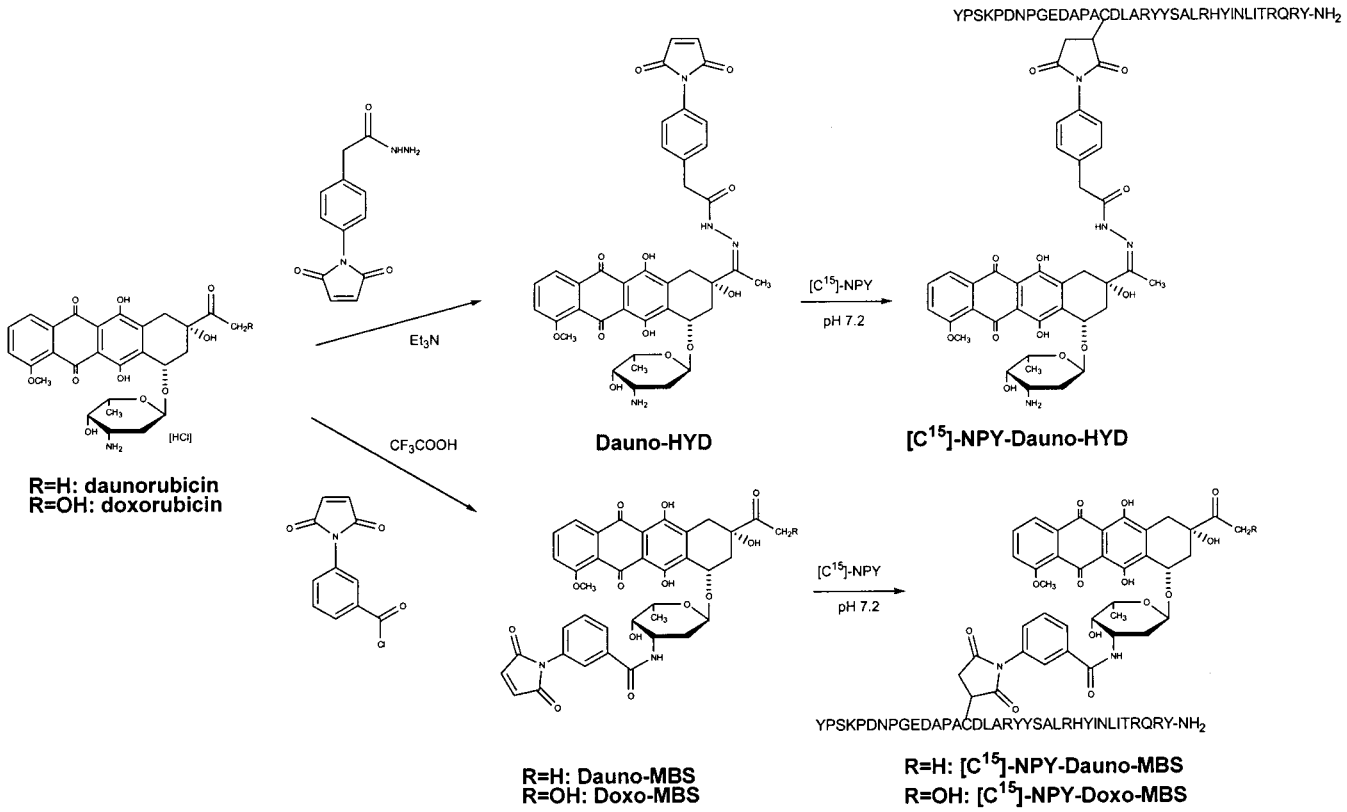

Figure 1. Synthesis of daunorubicin and doxorubicin maleimide derivatives.

receptor-mediated endocytosis.[18,19] Because structure−activity relationships (SARs) of NPY are well-known,[20,21] position 15 of NPY was used for attaching maleimide anthracycline derivatives which would presumably not lead to a significant loss of binding activity for the Y_1 receptor. Because the NPY−receptor complex is internalized and undergoes a pH shift from 7.4 to approximately 5.0 during endosomal trafficking, two different anthracycline derivatives that differ in the acid sensitivity of the bond between the drug and the spacer were selected (see Figure 1). Doxo-MBS and Dauno-MBS are characterized by a stable amide bond at the 3′-amino position of the anthracycline; Dauno-HYD is a daunorubicin derivative incorporating an acid-sensitive hydrazone linker at the 13-keto position. The maleimide moiety was introduced into daunorubicin and doxorubicin in order to selectively label the sulfhydryl group of [C^{15}]-NPY.

The conjugates [C^{15}]-NPY-Dauno-HYD, [C^{15}]-NPY-Dauno-MBS, and [C^{15}]-NPY-Doxo-MBS were synthesized, and binding assays were performed in the NPY Y_1 receptor expressing tumor cell line SK-N-MC. Furthermore, the in vitro cytotoxic effects of the compounds were tested by a colorimetric cellular cytotoxicity assay. As a control, receptor blocking experiments were performed, and cytotoxicity was investigated against glioblastoma XF-498L cells, which do not express NPY receptors. To gain insights into the localization and distribution of NPY, daunorubicin, and the prepared conjugates within the cell, confocal laser scanning microscopy studies were performed.

Experimental Section

Abbreviations: CF-NPY, carboxyfluorescein-neuropeptide Y; CLSM, confocal laser scanning microscopy; PBS, phosphate-buffered saline; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-5[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide.

Materials. Fmoc-protected amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin were obtained from NovaBiochem (Läufelfingen, Switzerland), diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole (HOBt), *N*-ethyldiisopropylamine (DIPEA), trifluoroacetic acid, thioanisole, 1,2-ethanedithiole and piperidine from Fluka, and *tert*-butyl alcohol from Merck. Acetone, dimethylformamide, diethyl ether and tetrahydrofuran were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). Daunorubicin HCl was a gift from Rhône Poulenc Rorer. Bovine serum albumin, XTT and bacitracin were purchased from Sigma, minimum essential medium with Earle's salts (MEM), RPMI 1640 medium, fetal calf serum, trypsine-EDTA, Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, glutamine, sodium pyruvate, and nonessential amino acids were from Gibco. Hoechst 33342 was obtained from Molecular Probes. EDTA, menadione and Pefabloc SC were from Fluka. [^{3}H]Propionyl-NPY (specific activity of 2.66 TBq/mmol) was purchased from Amersham.

Synthesis. The peptide [C^{15}]-pNPY was synthesized by automated multiple solid-phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum, Germany). To obtain the peptide amide, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin was used. The polymer matrix was polystyrene−1%-divinylbenzene (30 mg, 15 μmol). The side chain protection was chosen as follows: Tyr(*tert*-butyl), Glu(*tert*-butyl), Arg(2,2,3,5,5-pentamethylchromansulfonyl), His(trityl), Gln(trityl), Asn(trityl), Thr(*tert*-butyl), and Lys(*tert*-butyloxycarbonyl). Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxybenzotriazole activation, 10-fold excess and a coupling time of 40 min. The peptide amide was cleaved with 1 mL of trifluoroacetic acid/thioanisole/ethanedithiole (90:7:3) within 3 h. The peptide was precipitated from cold diethyl ether, collected by centrifugation and lyophilized from water/*tert*-butyl alcohol (1:1). Analysis of the product was performed by analytical reversed-phase HPLC at 220 nm on nucleosil C-18 columns from Merck-Hitachi (5 μm,

3 × 125 mm). Acetonitrile/water mixed with 0.1% trifluoroacetic acid was used as eluent. A flow rate of 0.6 mL/min and a gradient of 25−75% acetonitrile over 30 min was applied. Correct mass was identified by ion-spray mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany).

Maleimide derivatives of daunorubicin and doxorubicin (Dauno-MBS, Doxo-MBS, and Dauno-HYD) were prepared as described previously.[22,23] For the conjugation of [C^{15}]-NPY and the maleimide derivatives, the peptide was dissolved in water (1.19 mM concentration), Dauno-HYD in water/*tert*-butyl alcohol (1:1) (2.38 mM), Dauno-MBS in tetrahydrofuran (2.38 mM) and Doxo-MBS in dimethylformamide (2.38 mM). The coupling of the Dauno-MBS and Doxo-MBS moiety was performed with a 2-fold molar excess and a 4-fold for Dauno-HYD. PBS was added to maintain a pH value of 7.2. A typical reaction mixture contained 400 μL aqueous solution of [C^{15}]-NPY, 400 μL organic MBS compound (800 μL HYD, respectively), and 530 μL PBS. The reaction solutions were mixed, homogenized and stirred for 1 h at room temperature. The samples were centrifuged for 5 min (8000*g*, room temperature), the supernatants were evaporated in a speedvac (Variant S100) and dissolved in water/acetonitrile (1:1). The crude reaction mixtures were purified by preparative HPLC.

A RP C-18 column from Waters (6 μm, 25 × 300 mm) was used and acetonitrile with 0.08% trifluoroacetic acid and water as eluent. The products were separated with a flow rate of 2.5 mL/min and a gradient of 30−65% acetonitrile in 30 min. Fractions were collected and the solvent removed by the speedvac. After resuspension in water/*tert*-butyl alcohol (1:1), the samples were lyophilized. Identification was performed by ion-spray mass spectrometry, and purity was confirmed by analytical HPLC (λ = 220 nm).

Cell Culture. SK-N-MC cells were grown in MEM with Earl's salts containing 10% fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate and 1% nonessential amino acids. Cells were grown to confluency at 37 °C and 5% CO_2. The growth medium was removed and the cells were washed with Dulbecco's PBS. After incubation at room temperature for 3 min with PBS containing 0.02% EDTA, the cells were detached by mechanical agitation and suspended in new medium or incubation buffer. Glioblastoma cells XF-498L (kindly provided by H. H. Fiebig, Tumor Biology Center Freiburg) were grown in RPMI 1640 medium containing 10% fetal calf serum at 37 °C and 5% CO_2. At confluency, the cells were washed with Dulbecco's PBS and incubated at 37 °C for 5 min with trypsine-EDTA. Subsequently, the cells were detached and resuspended in new medium.

Receptor Binding Studies. Binding assays on SK-N-MC cells were performed (n = 3) using the following incubation buffer: MEM with Earl's salts containing 0.1% bacitracin, 50 μM Pefabloc SC and 1% bovine serum albumin.[24] Cells were washed with PBS and resuspended in incubation buffer. 200 μL of the suspension containing 400 000 cells were incubated with 25 μL 10 nM [^{3}H]propionyl-NPY and 25 μL 10 pM−10 μM [C^{15}]-NPY or conjugates. Nonspecific binding was defined in the presence of 1 μM unlabeled NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 2000*g* and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS, transferred to scintillation vials and mixed with scintillation cocktail. Radioactivity was determined using a β-counter (Beckman LS6500).

Colorimetric Cellular Cytotoxicity Studies. Cytotoxic activities of the conjugates were tested by a XTT-based colorimetric cellular cytotoxicity assay [25] (n = 3). SK-N-MC and glioblastoma XF-498L cells were grown to subconfluency in 96-wells. Growth medium was removed, and the cells were incubated with the peptide conjugates. The compounds had been dissolved in growth medium, concentrations ranged from 10^{-6} to 10^{-9} M. In addition to the cytotoxic conjugates, [^{15}C]-NPY and free daunorubicin were tested. Preincubation with BIBP 3226 (10^{-4} M) was performed for 3 h prior to the addition of daunorubicin or [C^{15}]-NPY-Dauno-HYD. Background was defined by the medium, and untreated cells were used for reference.

Table 1. Analytical Data of Peptides and Conjugates

peptide	mass calcd	mass exptl	HPLC (min)	purity[c] (%)	yield (mg)
[C^{15}]-NPY	4226.8	4226.0	13.96[a]	96	64
[C^{15}]-NPY-Dauno-MBS	4953.0	4953.8	13.00[b]	94	1.8
[C^{15}]-NPY-Dauno-HYD	4981.0	4981.8	15.37[b]	89	1.1
[C^{15}]-NPY-Doxo-MBS	4968.7	4968.6	12.65[b]	95	2.1

Gradient systems: [a]25−75% acetonitrile with 0.1% trifluoroacetic acid within 30 min; [b] 30−65% acetonitrile with 0.1% trifluoroacetic acid within 30 min. [c]According to HPLC. The detectable contaminants were mainly unconjugated [C^{15}]-NPY and, to a much smaller amount, incomplete adducts of Dauno-MBS, Doxo-MBS or Dauno-HYD and [C^{15}]-NPY (<2%).

Cells were incubated for 2 days under normal growth conditions and cytotoxicity subsequently measured with the XTT assay. Additionally, cell viability was confirmed by trypan blue exclusion. The XTT solution was prepared as follows: 5 mg XTT was dissolved in 5 mL MEM with Earl's salts at 37 °C, and 1.8 mg menadione was dissolved in 1 mL acetone. Prior to use, 50 μL of the menadione solution were added to the preheated MEM solution. From this final XTT solution, 50 μL was distributed in each well. After incubation at 37 °C and 5% CO_2 for 2 h, absorption was measured at 450 nm (Spectrafluor plus, Tecan).

CLSM Microscopy Studies. SK-N-MC cells were grown on a Chamber slide (Nunc Lab-Tek) for 5 days. Growth medium was removed, and the cells were incubated with carboxyfluorescein-neuropeptide Y (CF-NPY), [C^{15}]-NPY-Dauno-HYD, [C^{15}]-NPY-Dauno-MBS and daunorubicin, respectively. CF-NPY was prepared as described previously.[26] The compounds were dissolved in growth medium at concentrations of 1 μM, and untreated cells were used as reference. The nuclei of the cells were stained with Hoechst 33342 (final concentration: 1.76 μM). After incubation for 8 h at 37 °C, the cells were washed twice with prewarmed PBS, covered with fresh PBS and investigated unfixed under the microscope. A Zeiss LSM 410 inverted microscope was used (lasers: HeNe 543 nm, Ar 488/514 nm, and Ar UV 364 nm). Optical sections at intervals of 0.3 μm were taken with a 63 ×/1.3 Plan-Apochromat objective. Image processing was performed on a Silicon Graphics workstation using IMARIS, a 3D multichannel image processing software for confocal microscopic images (Bitplane AG, Zurich, Switzerland).

Results

Synthesis and Characterization. To attach pNPY to the anthracyclines, we modified the peptide and introduced a Cys in position 15, replacing the native Glu. [C^{15}]-NPY was synthesized by solid-phase peptide synthesis using Fmoc strategy, yielding the peptide with 96% purity. The sulfhydryl group was used to bind selectively to the carbon double bond of the maleimide moiety attached to daunorubicin and doxorubicin. The coupling step performed in solution turned out to be pH-sensitive with the fastest reaction and the highest yields being achieved at pH 7.2 in the presence of 20−30% organic solvent. 2−4-Fold excess of maleimide derivatives compared to the peptide was used. Coupling of the MBS moiety usually took place with better yields (30−38% according to HPLC) than coupling of the HYD moiety (19%). Analytical data of [C^{15}]-NPY and the prepared conjugates are shown in Table 1.

Receptor Binding Studies. Neuroblastoma cells SK-N-MC have been shown to express NPY Y_1 receptors.[27] Affinities of the new compounds were determined by displacement of [^{3}H]propionyl-NPY. The NPY ana-

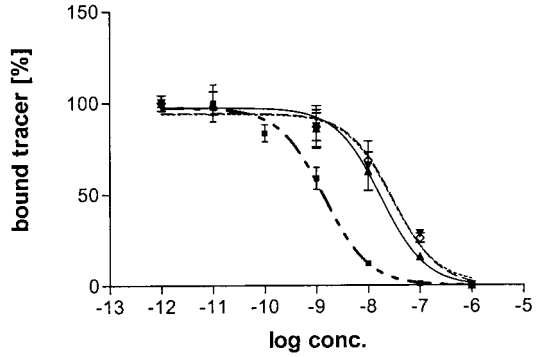

Figure 2. Binding of conjugates on SK-N-MC cells. Binding curves of [C15]-NPY (■; — – –), [C15]-NPY-Dauno-HYD (▲; —), [C15]-NPY-Dauno-MBS (▼; - - -), and [C15]-NPY-Doxo-MBS (◇; – – –).

logue [C15]-NPY shows high affinity (IC_{50} value 1.4 ± 0.2 nM) for this receptor. After conjugation of the anthracycline maleimide derivatives with the peptide, binding is reduced: The conjugates are still able to bind to the receptor; however, IC_{50} values are [C15]-NPY-Dauno-HYD = 24.9 ± 3.4 nM, [C15]-NPY-Dauno-MBS = 43.1 ± 11.2 nM, and [C15]-NPY-Doxo-MBS = 50.8 ± 9.8 nM. Displacement curves are shown in Figure 2.

Colorimetric Cellular Cytotoxicity Studies. The effect of the compounds on the target cells was investigated by a colorimetric cellular cytotoxicity, in which the tetrazolium salt XTT is bioreduced to a colored formazan.[28] Metabolism of the tetrazolium compound is therefore directly related to the cell number and indicates cell viability.

SK-N-MC cells expressing the Y_1 receptor were incubated with a 1 μM solution of the three conjugates, [C15]-NPY, and daunorubicin. Viability was determined after 48 h (Figure 3A). [C15]-NPY, [C15]-NPY-Dauno-MBS, and [C15]-NPY-Doxo-MBS showed no or only marginal effects. In contrast, [C15]-NPY-Dauno-HYD was as effective as free daunorubicin with respect to cytotoxicity, and the compounds were able to reduce cell growth by 66.9 ± 2.5% and 68.6 ± 0.4%, respectively. Pretreatment with 100 μM of the Y_1 receptor antagonist BIBP 3226[29,30] antagonized the cytotoxicity of [C15]-NPY-Dauno-HYD (cell viability remained at 89.1 ± 4.6%), but not that of daunorubicin. To investigate the minimal required concentration of the administered conjugates, dilutions were made and tested on SK-N-MC cells; the cytotoxic effects of [C15]-NPY-Dauno-HYD and daunorubicin were visible starting at 1 μM concentrations (Figure 3B). The same compounds were tested on glioblastoma XF-498L cells that do not express NPY receptors.[31] Daunorubicin was able to reduce cell growth by 47.3 ± 5%, whereas [C15]-NPY-Dauno-HYD showed no cytotoxicity (Figure 3C). This result indicates that selective tumor targeting is possible with [C15]-NPY-Dauno-HYD, because growing cells that do not express NPY receptors are not affected by the peptide conjugate in contrast to the nonconjugated chemotherapeutic drug.

Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy (CLSM) was used to study the intracellular distribution of NPY, daunorubicin, and the prepared conjugates. CLSM is a suitable technique to study the intracellular fate of the autofluorescent drug daunorubicin.[10] SK-N-MC cells were incubated with the compounds for 8 h and investigated under the microscope. To avoid nonspecific uptake into the nucleus, cells were investigated without fixation and permeabilization. Cell nuclei were stained with Hoechst 33342. Results are shown in Figure 4 (left-hand side: 3D-reconstructions; right-hand side: z-projections of the same pictures). Cell nuclei are shown in cyan, and the different compounds are depicted in red. The unconjugated peptide, CF-NPY, remained localized at a certain distance from the nuclei (Figure 4A) and is not distributed throughout the cells. The same pattern was also found with [C15]-NPY-Dauno-MBS (Figure 4B), indicating that the detectable Dauno-MBS is still coupled to the [C15]-NPY. In contrast, [C15]-NPY-Dauno-HYD (Figure 4C) as well as free daunorubicin (Figure 4D) were found in the perinuclear area (arrow). This might indicate that the cytotoxic active part Dauno-HYD is cleaved from the mother compound [C15]-NPY-Dauno-HYD by hydrolysis. As a consequence a similar distribution pattern is found as for free daunorubicin.

These results are in good agreement with the cytotoxic effects. Only the compound with the acid labile spacer HYD is active, whereas conjugates with an MBS spacer show no cytotoxicity.

Discussion

Coupling of anticancer drugs to macromolecular carriers has been shown to be a suitable approach to circumvent toxic side effects of these drugs to normal cells and to improve their efficacy toward malignant cells.[32,33] Polymers such as antibodies, serum proteins, polysaccharides, or synthetic copolymers accumulate in tumor tissue because of enhanced vascular permeability of tumor blood vessels and are retained there because of poor lymphatic drainage systems.[34,35] Compared to this passive tumor targeting, a more active one would be desirable by using selective receptors on the tumor cells or on tumor blood vessels.[36,37] The receptor targeting approach has become very important in nuclear oncology, and radiolabeled peptides are widely used for scintigraphy or radiotherapy.[38−43] Nevertheless, radiopeptides are able to perform their functions without necessarily entering the cells, whereas chemotherapeutic drugs have to be guided into the cell and subsequently released from the peptide carrier. Different peptides have been shown to be able to transport drugs through cell membranes, but this cellular uptake is usually receptor-independent, hence not specific.[14,44,45] By developing peptide−anthracycline conjugates that target receptors that are overexpressed on the distinct tumor cells, we made a first step toward receptor-mediated selective chemotherapy, where we could improve selectivity and reduce side effects. In principle, any peptide with specific receptors on the target can be used as a vector, as long as the peptidic conjugate retains high affinity for its receptor. Phage display strategy could be used to identify peptides capable of binding known target molecules or tissues.[15,46] An early attempt for peptide-guided chemotherapeutic tumor targeting has been done by Rigaudy,[47] whereas Schally and co-workers[48] and recently Huang[49] underlined this suitable approach. In our case, we chose NPY as a model peptide, as its receptors are overexpressed in a number of neuroblastoma. Although NPY receptors are naturally produced in the nervous system and the brain as

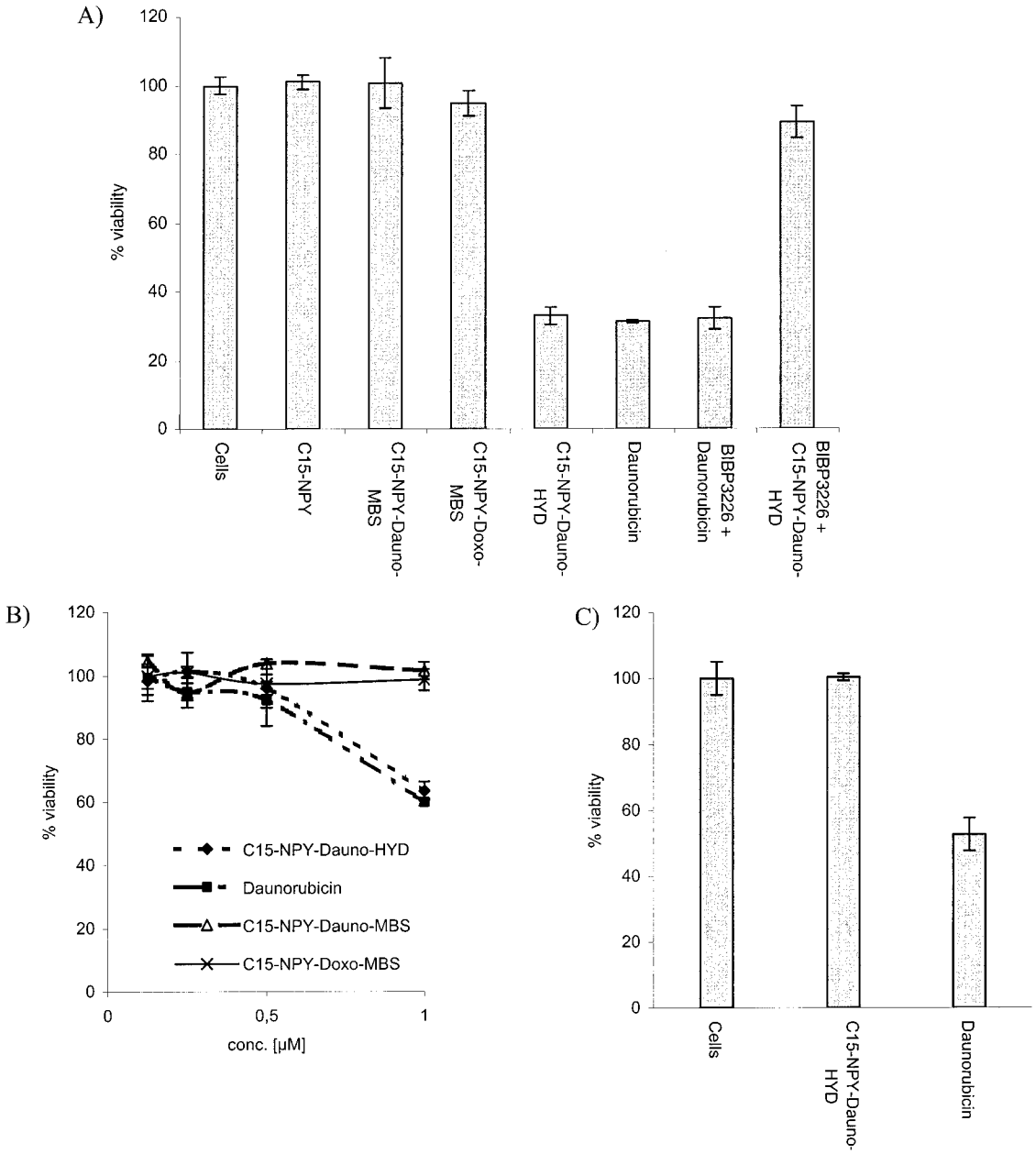

Figure 3. Cytotoxicity of conjugates on SK-N-MC cells (A, B) and glioblastoma cells (C). Viability of cells after incubation with compounds measured by a colorimetric cytotoxicity assay. In A and C, 1 μM concentration was administered for 48 h; in B, concentrations from 0.1 to 1 μM were administered for 36 h. 100 μM BIBP 3226 was added 3 h prior to treatment with daunorubicin or [C^{15}]-NPY-Dauno-HYD.

well, the quantitative difference in receptors between normal and malignant cells can be exploited for selective chemotherapy. Since we knew from previous studies which amino acids of NPY are essential for retaining binding affinity to the Y_1 receptor,[50] we replaced Glu-15 by a Cys, which is then able to bind selectively to the carbon double bond of the maleimide moiety attached to daunorubicin and doxorubicin.[51] As expected, we lost only little binding activity for the Y_1 receptor by this strategy as shown in the receptor binding studies.

The efficacy of the prepared conjugates of NPY and daunoubicin or doxorubicin was tested in vitro by a colorimetric cytotoxicity assay using SK-N-MC cells expressing the Y_1 receptor. Of our three conjugates ([C^{15}]-NPY-Dauno-MBS, [C^{15}]-NPY-Doxo-MBS, and [C^{15}]-NPY-Dauno-HYD) only the latter showed cytotoxicity in this cell line and was as potent as free daunorubicin. As a negative control, blocking experiments with a 100-fold excess of the Y_1 receptor antagonist BIBP 3226[29,30] were performed, which antagonized the cytotoxicity of [C^{15}]-NPY-Dauno-HYD but not that of daunorubicin. Moreover, we evaluated the cytotoxicity of the potent compounds in glioblastoma XF-498L cells that do not express NPY receptors. Since only free daunorubicin was able to kill these cells, we were able to induce selectivity by our peptide carrier approach.

Our results show that the cytotoxic activity of the conjugates is correlated with the chemical link between the peptide and the drug because using the HYD spacer led to cell death, whereas the use of the MBS spacer showed no effect. The main difference between these two spacers lies in their acid stability. The pH sensitivity of the hydrazone bond in HYD and the amide bond in

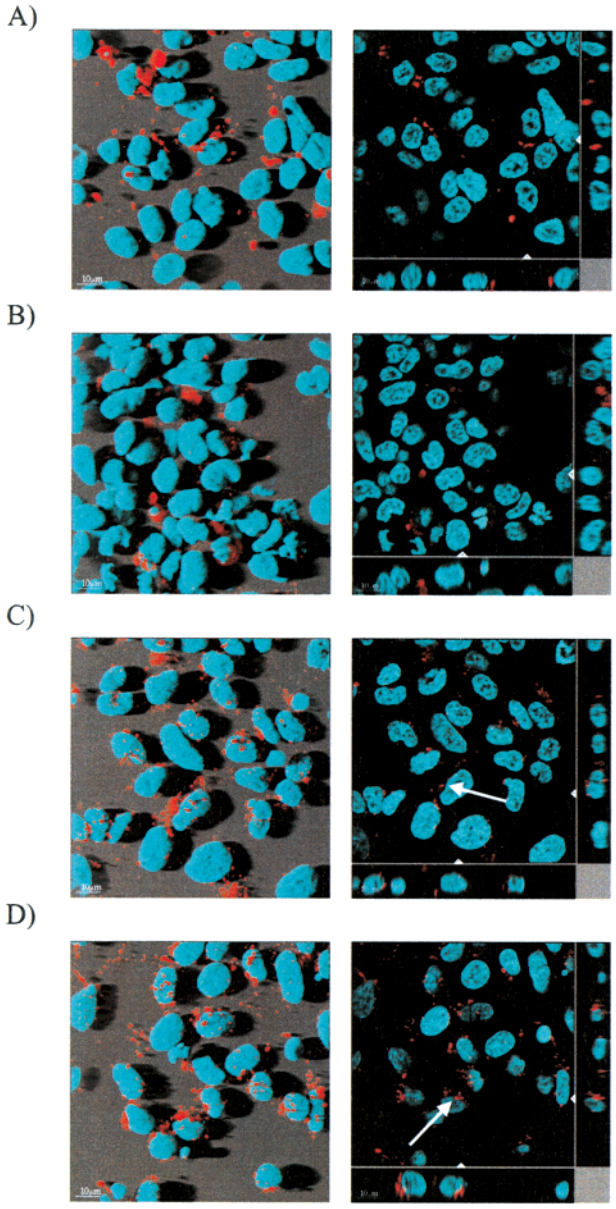

Figure 4. Visualization of conjugates on SK-N-MC cells by CLSM: 3D-reconstruction (left) and z-projection (right). Cell nuclei (cyan) are stained with Hoechst 33342, and compounds are shown in red: CF-NPY (A), [C^{15}]-NPY-Dauno-MBS (B), [C^{15}]-NPY-Dauno-HYD (C), and free daunorubicin (D).

MBS has been investigated previously demonstrating that the amide bond is stable at pH 7.4 and 5.0, whereas the hydrazone bond is stable at pH 7.4 but not at 5.0.[8,10,23] The hydrazone showed less than 10% degradation at pH 7.4 after 24 h, whereas at pH 5.0, a half-time of approximately 30 h was determined. Furthermore, ca. 90% stability in cell-conditioned medium or 10% FCS after 48 h has been found. This different behavior provides a tool for releasing the drug from its carrier in an acidic environment. During endocytosis, a significant drop of pH is observed: The extracellular space shows a physiological pH of 7.2−7.4, but in the endosomes the pH gradually drops from physiological to pH 5 in prelysosomes and subsequently to pH 4 in lysosomes.[18,52] Presumably, [C^{15}]-NPY-Dauno-HYD binds to the Y_1 receptor on the tumor surface and is internalized by receptor-mediated endocytosis,[19] and daunorubicin is released in the prelysosomes or lysosomes, from where it can diffuse into the cytoplasm and reach the target organelles. In contrast, the MBS conjugates will not release daunorubicin, and therefore no cytotoxic effects are observed. The importance of the acid sensitivity of the linker between the anticancer drug and the carrier for anticancer activity, which had been shown before,[5,22,52−55] could be confirmed.

The transport of doxorubicin into the cell by receptor-mediated endocytosis was shown to be a suitable approach for specific cytotoxicity by a research group at Bristol-Myers Squibb. Using acid-sensitive doxorubicin immunoconjugates with monoclonal antibodies, they could show antigen-specific antitumor activity in vitro and in vivo.[56] Furthermore, they realized that binding, internalization, and acid hydrolysis of the conjugate are required for specific cytotoxicity,[57] and with an optimized linker, complete cures of xenografted human carcinomas could be achieved.[58,59]

To further investigate the cellular uptake and distribution of our conjugates, CLSM was performed. The autofluorescent daunorubicin can be excited at 480 nm and shows a broad emission spectrum ranging from 500 to 680 nm.[11] Free NPY, applied as CF-NPY, and the MBS conjugate [C^{15}]-NPY-Dauno-MBS exhibited a similar distribution pattern. Both remain at a certain distance from the nucleus. In contrast, [C^{15}]-NPY-Dauno-HYD was enriched in the perinuclerar space and exhibited a similar distribution as free daunorubicin. This could be an indication that daunorubicin is released from the HYD conjugate within the cell and is subsequently able to perform its cytotoxic activity as free daunorubicin. In contrast, the MBS conjugate does not seem to be hydrolyzed, since it still shows the same localization as the carrier peptide NPY.

In conclusion, our studies demonstrate the potential application of suitable peptides for tumor targeting approaches and underline the importance of the nature of the chemical link between the drug and the carrier for cytotoxic activity.

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