Integrated Systems and Technologies

Development of Novel ADCs: Conjugation of Tubulysin Analogues to Trastuzumab Monitored by Dual Radiolabeling

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Abstract

Tubulysins are highly toxic tubulin-targeting agents with a narrow therapeutic window that are interesting for application in antibody–drug conjugates (ADC). For full control over drug–antibody ratio (DAR) and the effect thereof on pharmacokinetics and tumor targeting, a dual-labeling approach was developed, wherein the drug, tubulysin variants, and the antibody, the anti-HER2 monoclonal antibody (mAb) trastuzumab, are radiolabeled. 131I-radiodination of two synthetic tubulysin A analogues, the less potent TUB-OH (IC50 > 100 nmol/L) and the potent TUB-OMOM (IC50 ~1 nmol/L), and their direct covalent conjugation to 89Zr-trastuzumab were established. Radiodination of tubulysins was 92% to 98% efficient and conversion to N-hydroxysuccinimide (NHS) esters more than 99%; esters were isolated in an overall yield of 68% ± 5% with radiochemical purity of more than 99.5%. Conjugation of 131I-tubulysin–NHS esters to 89Zr-trastuzumab was 45% to 55% efficient, resulting in ADCs with 96% to 98% radiochemical purity after size-exclusion chromatography. ADCs were evaluated for their tumor-targeting potential and antitumor effects in nude mice with tumors that were sensitive or resistant to trastuzumab, using ado-trastuzumab emtansine as a reference. ADCs appeared stable in vivo. An average DAR of 2 and 4 conferred pharmacokinetics and tumor-targeting behavior similar to parental trastuzumab. Efficacy studies using single-dose TUB-OMOM–trastuzumab (DAR 4) showed dose-dependent antitumor effects, including complete tumor eradications in trastuzumab-sensitive tumors in vivo. TUB-OMOM–trastuzumab (60 mg/kg) displayed efficacy similar to ado-trastuzumab emtansine (15 mg/kg) yet more effective than trastuzumab (60 mg/kg). Our findings illustrate the potential of synthetic tubulysins in ADCs for cancer treatment. Cancer Res; 74(20); 5700–10. ©2014 AACR.

Introduction

Continuing increases in the incidence of cancer, coupled with the limitations of traditional chemotherapy, such as systemic toxicity and rapid development of multidrug resistance (1), have made the development of new therapeutic strategies a high priority. One of the most promising recent developments is targeted therapy, where drugs or other compounds are used that specifically interfere with targets involved in tumor growth and progression. Monoclonal antibodies (mAb) are a good example of targeted therapy and can also be used as a vehicle for toxic compounds, to form antibody–drug conjugates (ADC; ref. 2). The ideal ADC allows application of highly toxic compounds otherwise having exceedingly narrow therapeutic windows and exhibits higher tumor toxicity than current chemotherapeutics (3). In this context, the chemical synthesis of naturally occurring highly potent compounds and their derivatives becomes an important topic in ADC development. A very promising family of such compounds is represented by the tubulysins, which are tetrapeptides produced in small quantities by myxobacteria strains. The cytotoxic activity of tubulysins stems from their ability to inhibit tubulin polymerization and to disintegrate microtubules of dividing cells. Naturally occurring tubulysins have IC50 values between 0.01 and 10 nmol/L and can be chemically synthesized (ref. 4; for a comprehensive list of references see ref. 5). Over the last decade, various tubulysin analogues with different cytotoxic potency were synthesized (5, 6). Preclinical studies showed that tubulysins are too toxic for use as single-agent chemotherapeutics (7), and therefore targeted therapy becomes an appealing option to enable the administration of higher doses of tubulysins,
thus avoiding or minimizing systemic toxicity. Combining targeted therapy of mAbs with toxic agents might in fact result in highly specific and well-tolerated ADCs (8).

Over the last few years, several ADCs have entered phase II/III clinical trials. Two of these, brentuximab vedotin and ado-trastuzumab emtansine (Adcetris and Kadcyla = T-DM1), are U.S. Food and Drug administration (FDA)–approved and are now used to treat patients with Hodgkin disease and HER2-positive (human epidermal growth factor receptor 2) metastatic breast cancer, respectively. These and most of the other ADCs still in development consist of chimeric, humanized or fully human mAbs conjugated to tubulin-targeting inhibitors or DNA-damaging agents (9).

Most ADCs consist of three components: the mAb, the toxic compound and a linker that combines the two parts. Three types of linkers are used to couple the mAb and the toxic compound: releasable, cleavable, and noncleavable linkers (10). Releasable linkers form a relatively stable ADC in serum and release the drug upon internalization in the cell. Accordingly, selective tumor release is due to the difference in composition between plasma and cytosol. Examples of releasable linkers are hydrazones and disulfides (11–14). ADCs containing a cleavable linker are about 100 times more stable in serum compared with ADCs with a releasable linker. An example is the peptide based p-aminobenzyl carbamate linker, which is used in the clinically available ADC brentuximab vedotin (15, 16). Noncleavable linkers result in the most stable ADCs. Only upon internalization of the complete ADC and after degradation of the mAb the toxic part including an amino acid residue is released and can become active. Thioether bonds formed from SMCC [succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate] are reported to be noncleavable linkers. Ado-trastuzumab emtansine contains this SMCC-based linkage mandatory. Because we and others (17, 18) have shown that the thioether bond is not such a noncleavable bond as presumed, we chose not to use a linker and opted for a noncleavable bond that has found multiple applications in the clinic (17, 19), namely a direct amide bond formation of tubulysins with lysine residues of the mAb. In fact, tubulysins possess a C-terminal carboxylic acid group enabling direct coupling with these lysine residues after conversion to an active ester.

Before an ADC can be used in in vivo experiments, the stability of the conjugate must be confirmed. Also, knowledge of the pharmacokinetics can be informative for future development. For a thorough study of the targeting behavior of the ADCs and their in vitro and in vivo stability, and to facilitate monitoring of conjugation and purification procedures, we decided to radiolabel the tubulysin analogues as well as the mAb. In the present study, we describe the 131I-radioiodination of two synthetic tubulysin A analogues, the less potent TUB-OH and the potent TUB-OMOM, and the conjugation of these 131I-labeled tubulysins to nonlabeled or 89Zr-labeled trastuzumab (Fig. 1). The obtained ADCs were evaluated in vitro for effects on cell viability, and in vivo for...
their pharmacokinetic behavior and tumor-targeting potential. After determination of the maximum-tolerated dose (MTD), antitumor effects of the most potent ADC were tested in nude mice bearing trastuzumab-resistant JIMT or trastuzumab-sensitive N87 tumors using ado-trastuzumab emtansine as reference.

Materials and Methods

General materials and methods

Cell lines used were the breast cancer lines MDA-MB231, JIMT, and SKBR3, the ovarian cancer cell line SKOV, and the gastric cancer cell line N87. JIMT-1 was obtained from DSMZ Germany on March 19, 2012, after cytogenetic testing, and used within 6 months after resuscitation. NCI-N87 was obtained from ATCC United Kingdom on February 29, 2012, after cytogenetic testing, and used within 6 months after resuscitation. SKBR3 was obtained from Dr. T. Oude Munnink (Department of Medical Oncology, University Medical Center Groningen, Groningen, The Netherlands), MDA-MB-231 from Roche, and SK-OV-3 from the Department of Medical Oncology, VU University Medical Center Amsterdam. All cell lines were checked for primary growth characteristics (morphology and growth rate) and HER2 expression. MDA-MB231 is a cell line with low HER2 expression; SKBR3, SKOV, and N87 are overexpressing HER2; and JIMT is developed from tumor cells of a patient with trastuzumab resistance and is HER2-positive (20). 

131I (56.6 GBq/mL in 0.02 mol/L NaOH) and 89Zr (13.3 GBq/mL in 0.1 mol/L NaOH) was purchased from GE Healthcare or PerkinElmer, 124I (1.2 GBq/mL in 0.02 mol/L NaOH) and 86Sr (≥0.15 GBq/nmol in 1 mol/L oxalic acid) were obtained from PerkinElmer. The tubulysin analogues TUB-OH and TUB-OMOM were synthesized by KemoTech (www.kemotech.it). Trastuzumab and ado-trastuzumab emtansine were obtained from the hospital pharmacy. High-performance liquid chromatography (HPLC) analyses were performed using a Jasco HPLC system equipped with a Jupiter 4 µ Proteo 90A Column (Phenomenex) using a gradient of H2O/MeCN (both containing 0.1% TFA) as eluent at a flow rate of 0.5 mL/min for the analysis of the tubulysins and a Superdex 200 10/30 GL size exclusion column (GE Healthcare Life Sciences), using a mixture of 0.05 mol/L sodium phosphate, 0.15 mol/L sodium chloride (pH 6.8), and 0.01 mol/L Na2SO3 as the eluent at a flow rate of 0.5 mL/min for analysis of the mAb constructs. The radioactivity of the eluate was monitored using an inline NaI(Tl) radiodetector (Raytest Sockett). Single/double isotope counting was performed with a gamma-well counter (Wallac LKB-CompuGamma 1282; Pharmacia) for 131I, 124I, and 86Sr. In case of dual isotope measurements, cross-over corrections from one radionuclide into the alternate window were performed using a standard of each radionuclide.

Preparation of radioiodinated tubulysin esters

Tubulysin analogues TUB-OH (MW = 687 g/mol) or TUB-OMOM (MW = 745 g/mol; Fig. 1) were dissolved in H2O/MeCN (1/1) at a concentration of 3.1 µg/mL. Hundred microliters of TUB-OH (450 nmol) or TUB-OMOM (415 nmol) was incubated with 30 µL MeCN, 100 µL 0.5 mol/L phosphate buffer (pH 7.2), 3 to 100 MBq 131I (volume: 4–8 µL), and 25 µL (50 µg: 2 mg/mL) chloramine-T, by shaking in a Thermomixer at 550 rpm at room temperature. After 2 minutes, the reaction was stopped with 10 µL Na2 SO3 (20 µg: 20 mg/mL) and a 1-µL sample was taken and diluted to 20 µL with H2O/MeCN (7/3) for HPLC analysis of the radioiodinated tubulysin and assessment of radiolabeling efficiency. Unreacted 131I and reduced chloramine-T were removed by use of a Seppak TC2 Light column (Waters), via loading of the mixture on the column and washing with 10 mL H2O. Subsequently, 131I-TUB-OH or 131I-TUB-OMOM was eluted from the Seppak with 2 mL MeCN, thereafter the MeCN was evaporated under a nitrogen flow at 39°C. Next, 8 mg N-hydroxysuccinimide (NHS; Sigma-Aldrich; 0.07 mmol) in 500 µL MeCN was added to the dried 131I-TUB-OH or 131I-TUB-OMOM. The esterification was started by adding approximately 25 mg EDC [1-ethyl-3-[3-dimethylaminopropyl]carbodiimide; 0.16 mmol] as a solid. After 30 minutes in an ultrasonic bath, the reaction mixture was diluted 10-fold with H2O and trapped on a Seppak TC2 Light column. Unreacted/excess NHS and EDC side products were removed by washing with 25 mL H2O, after which 131I-TUB-OH-NHS or 131I-TUB-OMOM-NHS was eluted from the inverted Seppak column with 2 mL MeCN. After MeCN evaporation at 39°C under a nitrogen flow, the products were redissolved in 55 ± 5 µL MeCN and stored at −80°C.

Preparation of single- and dual-labeled ADCs

Trastuzumab (2 mg, 95 µL; 13.3 nmol; 21 mg/mL) was mixed with 0.9% sodium chloride (900 µL) and 50 mmol/L Na2CO3 (7 µL), after which 4 or 8 equivalents (eq; 53 or 106 nmol) of 131I-TUB-OH-NHS or 131I-TUB-OMOM-NHS in MeCN (10–20 µL) were added and incubated for 30 minutes at room temperature in a Thermomixer at 550 rpm. The ADC, 131I-TUB-OH-trastuzumab, or 131I-TUB-OMOM-trastuzumab containing on average either two or four tubulysin molecules per mAb molecule (2 eq, respectively, 4 eq), was purified using a PD-10 column (GE Healthcare) or HiTrap Desalting Column (GE Healthcare). Protein concentration was determined by HPLC using a calibration curve of trastuzumab. Instant thin layer chromatography (ITLC) analysis of the ADCs was carried out to assess the conjugation efficiency and radiochemical purity of the ADC. Silica-impregnated glass fiber sheets (PI Medical Diagnostic Equipment BV) were used with 20 mmol/L citrate buffer (pH 5.0)/MeOH (3/7) as the mobile phase. As readout gamma-well counting or phosphor imager analysis (FLA 8000; GE Healthcare) followed by quantification with ImageQuant software was used.

Dual-labeled 131I-TUB-OMOM-89Zr-trastuzumab was obtained from 89Zr-labeled trastuzumab (2 mg in 1 mL 0.9% NaCl, 13.3 nmol, 3.2 MBq 89Zr) following exactly the same procedure as described above. Premodification of trastuzumab (5 mg, D/mAb molar ratio 0.5) and labeling with 89Zr (10 MBq) was performed according to part 1 and 3 of the protocols described by Cohen and colleagues (21).
Stability and in vitro cell binding of the ADCs

ADCs 131I-TUB-OH–trastuzumab and 131I-TUB-OMOM–trastuzumab were stored at 4°C in 0.9% NaCl and at 37°C in 0.9% NaCl or 60% human serum. At different time points, radiochemical purity of the conjugates was measured by iTLC, while conjugate integrity was analyzed by SDS-PAGE followed by phosphor imager analysis. For SDS-PAGE, samples were mixed at 1:1 with loading buffer and run on a Phastgel System (GE Healthcare Life Sciences) using preformed 7.5% SDS-PAGE gels under nonreducing conditions. The gel was analyzed using a phosphor imager and quantified with ImageQuant software. In vitro binding characteristics of the ADCs were determined in an immunoreactivity assay essentially as described by Lindmo and colleagues (22), using a serial dilution of 0.2% glutaraldehyde-fixed N87 cells and a fixed amount of ADC 131I-TUB-OH–trastuzumab or 131I-TUB-OMOM–trastuzumab (5–15 ng). After overnight incubation at 4°C, the cell suspension was centrifuged and the specific binding calculated as the ratio of cell-bound radioactivity to the total amount of radioactivity in the assay. This was corrected for nonspecific binding, as determined with a 500-fold excess of nonradioactive trastuzumab. All binding assays were performed in triplicate.

In vitro cell viability assay

The effects of the tubulysins TUB-OH and TUB-OMOM, the ADCs 131I-TUB-OH–trastuzumab and 131I-TUB-OMOM–trastuzumab (both trace-dose 131I-labeled, to exclude radiation effects), and trastuzumab on cell viability of the cell lines MDA-MB231, JIMT, SKBR3, SKOV, and N87 were measured with the CellTiter-Blue Assay. The cells were trypsinized and plated in 96-well, flat-bottomed, tissue culture plates at day 0. On day 1 TUB-OH, TUB-OMOM, 131I-TUB-OH–trastuzumab, 131I-TUB-OMOM–trastuzumab, or trastuzumab were added at concentrations of 100, 10, 1, 0.1, and 0.01 nmol/L (in case of ADCs, concentration of the mAb was used). On day 5, the CellTiter-Blue reagent was added and incubated for 2 hours at 37°C, and viable cells were measured with a Tecan plate reader (Tecan Group Ltd.) at 560 nm. Fluorescence values of the samples were corrected for background of cell culture medium, the results, presented as the percentage of survival, were calculated by dividing the fluorescence values of the treated cells by the values of the untreated control cells.

Blood kinetics: 131I-TUB-OH–trastuzumab versus 124I-trastuzumab

For a first indication of the pharmacokinetic behavior and the in vivo stability of the conjugate, 131I-TUB-OH–trastuzumab was evaluated in non–tumor-bearing female nude mice (Hsd athymic nu/nu, 25–32 g; Harlan CPB) and compared with 124I-trastuzumab via coinjection. Heretofore, trastuzumab (250 μg) was reacted with 19 MBq 124I in the presence of 0.5 μg NaI as carrier according to Tijink and colleagues (23) Labeling efficiency was 96%, purification with PD10 resulted in a 99.9% pure 124I-trastuzumab. All animal experiments were performed according to the Dutch National Institutes of Health principles of laboratory animal care and Dutch national law ("Wat op de dierproeven," Stb 1985, 336). Mice were coinjected with 0.15 MBq 131I-TUB-OH–trastuzumab (2 eq; 86 μg mAb) and 0.3 MBq 124I-trastuzumab (4 μg mAb) in a total volume of 120 μL, intravenously. Blood was collected by tail laceration at 1, 2, 24, 48, 72, 96, and 168 hours p.i. Blood was weighed, and the amount of radioactivity was measured in a gamma-well counter with cross-over corrections. Radioactivity levels were calculated as the percentage of the injected dose per gram of tissue (%ID/g).

Biodistribution of dual-labeled 131I-TUB-OMOM–89Zr-trastuzumab conjugates

Biodistribution of 131I-TUB-OMOM–89Zr-trastuzumab (2 eq). 131I-TUB-OMOM–89Zr-trastuzumab (4 eq), and 89Zr-trastuzumab was compared in female nude mice (Hsd athymic nu/nu, 25–32 g; Harlan CPB), bearing N87 or JIMT tumors on both flanks. Mice were injected (i.v.) in a total volume of 100 μL with 0.3 MBq (0.15 MBq of each isotope) 131I-TUB-OMOM–89Zr-trastuzumab (100 μg mAb). At 3, 24, 48, or 72 hours after injection, the mice were anesthetized, bled, euthanized, and dissected. Blood and organs were weighed, and further processed as described above.

To evaluate the biodistribution of unconjugated tubulysin, 8 mice were injected (i.v.) with 0.12 MBq 131I-TUB-OMOM (1.7 nmol in a 3:7 mixture of ethanol and 0.9% NaCl, an equal molar amount of 131I-TUB-OMOM as received by mice injected with 131I-TUB-OMOM–trastuzumab, 2 eq). After 1, 2, 3, and 24 hours, 2 mice were anesthetized, bled, euthanized, and dissected and further processed as described above.

In vivo MTD and therapy study

Before starting the therapy study, the MTD for TUB-OMOM–trastuzumab was determined. For this purpose, five groups of 5 nude mice were given 15, 30, 45, or 60 mg/kg trace-dose–labeled 131I-TUB-OMOM–trastuzumab (4 eq) or normal saline as a control by an i.p. bolus injection. Trace-dose labeling in this and subsequent experiments was only used to confirm tubulysin-to-mAb molar ratio. Body weight was measured three times per week and MTD was reached when weight loss was >10% compared with the control mice.

The therapeutic effectiveness of trace dose–labeled 131I-TUB-OMOM–trastuzumab (4 eq) was studied in the same nude mice models as described for the biodistribution study. For this purpose, six groups of 7 mice with established JIMT or N87 xenografts in both flanks were used. The mean tumor size at the start of the study was 85 ± 15 mm3 and was similar for the different treatment groups. All mice received an i.p. bolus injection. Group 1 was the control group and received 200 μL of saline solution. Group 2, 3, and 4 received 15, 30, and 60 mg/kg of trace dose–labeled 131I-TUB-OMOM–trastuzumab (4 eq), respectively, group 5 received 15 mg/kg trastuzumab and group 6 received 15 mg/kg ado-trastuzumab emtansine. Ado-trastuzumab emtansine was included in this study as a reference ADC with proven clinical efficacy. Body weight and tumor volume were measured three times per week up to 3 months after end of treatment.
Statistical analysis
All animal experiments were statistically analyzed with SPSS, version 21 (SPSS Inc.) using the Student t test for independent samples. Two-sided significance levels were calculated, and \( P < 0.05 \) was considered statistically significant.

Results
Effects of TUB-OH and TUB-OMOM on in vitro cell viability
The toxicity of TUB-OH and TUB-OMOM was determined in the CellTiter-Blue Assays. Five different cell lines were incubated with TUB-OH or TUB-OMOM for 5 days. The HER2-overexpressing cell lines N87, SKBr3, and SKOV, the trastuzumab-resistant cell line JIMT, and the HER2-negative cell line MDA-MB231 were found to be relatively insensitive to TUB-OH (Supplementary Fig. S1A), the corresponding IC\(_{50}\)'s are >100 nmol/L. However, TUB-OMOM (Supplementary Fig. S1B) showed IC\(_{50}\) values between 0.4 and 6 nmol/L.

Preparation of radioiodinated tubulysin esters and their corresponding ADCs
The tubulysin analogues TUB-OH and TUB-OMOM are poorly soluble in aqueous solution and required the addition of MeCN to obtain a homogeneous solution. Radioiodination was carried out with \(^{131}\)I-iodide, using chloramine-T as oxidant. The reaction depicted in Fig. 1 was performed in a 7:3 (H\(_2\)O/MeCN) solution, resulting in a radiolabeling efficiency of 92% to 98% for \(^{131}\)I-TUB-OH and \(^{131}\)I-TUB-OMOM (Fig. 2A). Purified \(^{131}\)I-TUB-OH and \(^{131}\)I-TUB-OMOM were reacted with NHS in MeCN to form \(^{131}\)I-TUB-OH–NHS and \(^{131}\)I-TUB-OMOM–NHS with a conversion >99% (Fig. 2A). Via an inverted Seppak T2 Light purification procedure, the radioiodinated tubulysin esters were isolated in 68% ± 5% overall yield with a radiochemical purity of >99.5%. The NHS esters were stored in a glass vial at −80°C as a MeCN solution at a concentration of 5.3 nmol/\(\mu\)L. Esters were produced with three different \(^{131}\)I-specific activities, namely 114 ± 6, 57 ± 3, and 6 ± 1 kBq/nmol.

For preparation of single- and dual-labeled ADCs, 2 mg of trastuzumab bearing no radiolabel or labeled with 3 MBq \(^{89}\)Zr were incubated with 4 (114 kBq \(^{131}\)I/nmol) or 8 (57 or 6 kBq \(^{131}\)I/nmol) eq of \(^{131}\)I-TUB-OH–NHS or \(^{131}\)I-TUB-OMOM–NHS at pH ~9 for 30 minutes, resulting in 45% to 55% conjugation efficiency (TLC) and with retention of integrity (SDS-PAGE; Fig. 2B). Longer reaction times did not increase conjugation efficiency. Purification of the ADC was performed using size exclusion chromatography and resulted in a radiochemical purity of 96% to 98% (PD10 columns or HiTrap Desalting columns; Fig. 2B and C).

In vitro stability and effects on cell viability of ADCs
The stability of the ADCs was determined at 4°C in 0.9% aqueous NaCl and at 37°C in 60% human serum (0.9% NaCl included for comparison) by iTLC. At time point \( t = 0 \), a maximum binding between 93% and 96% to N87 cells. After 5 days, the binding assay showed a decrease of maximum binding to 89% to 92%. However, when binding data were corrected for release of \(^{131}\)I, no decrease of binding in time became apparent.
Radiolabeling Monitored Tubulysin to Trastuzumab Conjugation

Figure 3. In vitro cell viability assay. The effect of trastuzumab (black line), TUB-OH-trastuzumab (dashed black line), TUB-OMOM–trastuzumab (2 eq, trace dose-labeled; gray line), and ado-trastuzumab–emtansine (dotted black line) on viability of JIMT cells. Shown are representative experiments performed with six replicates per concentration and data are presented as mean ± SD.

Figure 3 shows the effect of trastuzumab, 131I-TUB-OH–trastuzumab, 131I-TUB-OMOM–trastuzumab, and ado-trastuzumab emtansine on the viability of JIMT cells. The tubulysin ADCs were only trace dose–labeled (6 kBq 131I/nmol). No growth inhibition effect was seen of 131I-TUB-OH–trastuzumab on JIMT cells; therefore, it is safe to say that the low dose of radioactivity does not have an influence on cell viability. Both 131I-TUB-OMOM–trastuzumab and ado-trastuzumab emtansine caused a decrease in the viability of the JIMT cells compared with trastuzumab alone, but the effect is not as strong as for TUB-OMOM alone (Supplementary Fig. S1A).

Pharmacokinetics and tumor targeting of ADCs with 0, 2, or 4 TUB molecules per mAb molecule

A pilot study was performed in non–tumor-bearing mice to evaluate the effect of tubulysin coupling on antibody pharmacokinetics. To this end, 125I-trastuzumab and 131I-TUB-OH–trastuzumab (2 eq) were co-injected to compare the in vivo behavior of the mAb. The blood kinetics did not show significant differences (P > 0.05): at 1 hour p.i. the blood levels were 29.6 ± 2.0 and 31.5 ± 2.3 %ID/g and slowly decreased to 6.7 ± 0.8 and 5.4 ± 1.0 %ID/g for 125I-trastuzumab and 131I-TUB-OH–trastuzumab, respectively, at 168 hours (Supplementary Fig. S2). These data indicate that coupling of tubulysins (2 eq) does not alter pharmacokinetics of trastuzumab, and that conjugates are stable in vivo in blood.

For a more detailed study of the effect of tubulysin coupling on pharmacokinetics and tumor targeting, N87 and JIMT tumor-bearing mice were injected with dual-labeled 131I-TUB-OMOM–89Zr-trastuzumab (0, 2, or 4 eq) or with unconjugated 131I-TUB-OMOM to evaluate the kinetics of free drug excretion. Figure 4 gives insight in the accumulation of the 2 eq (Fig. 4, left) and 4 eq-conjugate (Fig. 4, right) in the tumors and organs of N87-bearing mice for both 89Zr and 131I. At 3, 24, 48, and 72 hours p.i. of the 2 eq-conjugate, blood levels for 131I were not significantly different from 89Zr, confirming stability of the conjugate in blood. The tumor uptake values for 131I were 7.8 ± 1.9, 18.6 ± 3.8, 16.6 ± 4.0, and 12.4 ± 6.2 %ID/g and are lower than those of 89Zr (8.4 ± 2.0, 18.8 ± 2.4, 20.5 ± 4.7, and 21.2 ± 4.6 %ID/g). This is most probably due to the fact that 131I is released from the tumor cells after internalization, while 89Zr residuals inside the cells. At 3 hours p.i., no significant differences in 131I and 89Zr uptake in normal tissues were observed, except for colon and ileum content where 131I showed higher levels than 89Zr: 7.7 ± 4.5 versus 0.6 ± 0.3 %ID/g in case of colon content and 2.3 ± 0.6 versus 0.2 ± 0.3 %ID/g in case of ileum content. Like for tumors, also for normal tissues 131I and 89Zr showed diverging uptake levels from 24 hours p.i. on, with differences becoming larger at later time points after injection of 131I-TUB-OMOM–89Zr-trastuzumab. Similar trends in biodistribution were observed for 131I-TUB-OMOM–89Zr-trastuzumab (4 eq) at N87 tumor–bearing nude mice (Fig. 4B) and for 131I-TUB-OMOM–89Zr-trastuzumab (2 and 4 eq) in JIMT-tumor–bearing nude mice (Supplementary Fig. S3). Of note, in both tumor models 131I-TUB-OMOM-conjugated 89Zr-trastuzumab (2 and 4 eq) did not show significant differences in blood levels as well as tumor uptake levels when compared with unconjugated 89Zr-trastuzumab (Supplementary Fig. S4).

We postulated that increased levels of 131I in colon and ileum content might originate from a small proportion of unconjugated 131I-TUB-OMOM present in the preparation for injection, rather than from free 131I, as stomach content did not contain elevated levels of 131I. To test this hypothesis, 131I-TUB-OMOM was administered to tumor-free mice. Figure 5 confirms that free 131I-TUB-OMOM shows rapid blood clearance and predominant accumulation in the colon and ileum content.

In vivo MTD and therapy study

A MTD study was performed to determine the MTD of TUB-OMOM–trastuzumab (4 eq). Nontumor-bearing mice were injected with 15, 30, 45, or 60 mg/kg, single bolus injection. No weight loss or other signs of toxicity were observed. Next, the in vivo efficacy of different doses of TUB-OMOM–trastuzumab was compared with trastuzumab and ado-trastuzumab emtansine in JIMT- and N87-tumor–bearing mice. JIMT tumors showed no significant growth delay when injected with 15, 30, or 60 mg/kg TUB-OMOM–trastuzumab or 15 mg/kg ado-trastuzumab emtansine compared with the mice injected with trastuzumab alone (Supplementary Fig.
Figure 6 shows that mice with N87 tumors responded when injected with trastuzumab, but to a distinct lesser extent than after injection of the ADCs. Tumor response upon treatment with TUB-OMOM–trastuzumab was dose dependent with the highest dose, 60 mg/kg, showing the same effect as ado-trastuzumab emtansine, 15 mg/kg. Cures, defined as no outgrowth of regressed individual tumors during the follow-up period, were seen in mice treated with 30 mg/kg TUB-OMOM–trastuzumab (n = 1), 60 mg/kg TUB-OMOM–trastuzumab (n = 3), and 15 mg/kg ado-trastuzumab emtansine (n = 3).
Discussion

Stability in blood is a prerequisite in the development of ADCs when highly toxic compounds like tubulysins are used, because sequestration of the free drug in normal organs might cause unacceptable toxicity. In addition, it is important to determine whether the tumor-targeting capacity of a mAb is preserved upon coupling of such drugs and to gain knowledge on antitumor effects as efficacy strongly depends on ADC internalization and intracellular processing. Most studies focus on the antitumor effects of the ADC, while neglecting thorough characterization of the ADC in vivo. The easiest way to get this information is by using a strategy of dual-radiolabeling in which drug and mAb each receive a different radiolabel.

In this study, we developed procedures to radiolabel the less potent TUB-OH (IC50 > 100 nmol/L) and the potent TUB-OMOM (IC50, 0.4–6 nmol/L) with 131I, to convert them to an active NHS ester, and to couple the esters successfully to unlabeled or 89Zr-labeled trastuzumab. Conversion of the 131I-labeled tubulysins to the NHS esters was >99%, while the esters were isolated at an overall yield of 68% ± 5% and a radiochemical purity of >99.5%. Conjugation of the 131I-tubulysin esters to unlabeled or 89Zr-labeled trastuzumab antibody was 45% to 55% efficient. Because all tubulysin analogues possess a C-terminal carboxylic acid group, the conjugation procedure developed herein is generally applicable for the production of tubulysin ADCs. Radiochemical purity of the radiolabeled ADCs was 96% to 98%, which leaves room for further optimization.
improvement when tubulysin-antibody conjugates are prepared for clinical application.

Several radioiodination and esterification routes were explored before arriving at the current procedure. The described mild radioiodination route makes use of chloramine-T as the oxidant, the presence of MeCN in the reaction mixture prohibited the use of iodogen-coated vials (24). The tubulysins required the presence of a free phenol group on the C-terminal amino acid fragment, tubulysins with a methoxyphenyl group could not be radioiodinated. 131I-TUB-OH and TUB-OMOM were reacted with NHS, sulfoNHS, and TFP-OH, only the NHS ester could be efficiently formed and purified by Seppak. The TFP-ester did not elute from the Seppak (too apolar) and sulfoNHS-ester was not trapped on the Seppak (too polar). The radioiodine label also revealed that upon purification with size exclusion chromatography, about 2% to 4% of nonconjugated tubulysin coeluted with the ADC, possibly because of trapping in the tertiary structure of the mAb molecule.

Radioiodination of the tubulysins provided us with extra information about the in vitro and in vivo stability of the ADCs and their in vivo tumor-targeting characteristics. To remain within the same drug–antibody ratio (DAR) as ado-trastuzumab emtansine (DAR, 3–4), in this study, on average 2 or 4 eq of 131I-TUB-OH or 131I-TUB-OMOM were coupled per trastuzumab molecule (25). Storage of the ADCs for 5 days in NaCl at 4°C did not result in release of 131I, while 3% to 4% release of 131I was observed when ADCs were stored in serum or NaCl at 37°C for 5 days. No impairment of ADC binding to target cells was observed under these storage conditions. Good stability of ADCs was also confirmed in vivo, comparison of the blood kinetics of 131I-trastuzumab and 131I-TUB-OMOM (Supplementary Fig. S2) showed no significant differences.

The hydrophobicity of a coupled drug, as well as the type, number, and arrangement of charged groups, can strongly influence the physicochemical properties of the mAb, resulting in extended aggregation, altered pharmacokinetics, and impairment of tumor targeting, even when the in vitro antigen binding of the mAb remains preserved (26–28). For this reason, conjugation procedures are currently introduced that give optimal control over the number of drug molecules coupled to the mAb as well as the mAb sites for drug coupling (29–31). In our studies, 0, 2, or 4 eq of 131I-TUB-OMOM on average were directly coupled to the lysine residues of 89Zr-trastuzumab, avoiding premodification of the mAb as in case of ado-trastuzumab emtansine (12). At 3, 24, 48, and 72 hours after administration of the 131I-TUB-OMOM–89Zr-trastuzumab (2 or 4 eq) conjugates to N87- or JIMT-tumor-bearing mice, blood levels for 131I were in general not significantly different from 89Zr, confirming stability of the conjugates in blood. What is more, in both tumor models blood levels as well as tumor uptake of antibody as measured by 89Zr counting were the same for 131I-TUB-OMOM–89Zr-trastuzumab (2 or 4 eq) and 89Zr-trastuzumab, indicating that coupling of TUB-OMOM to trastuzumab does not alter the pharmacokinetics and tumor-targeting properties of trastuzumab (inert coupling).

Use of dual-radioabeled 131I-TUB-OMOM–89Zr-trastuzumab conjugates in vivo led to two other important observations: (i) elevated uptake of 131I in comparison with 89Zr in colon and ileum content, most probably due to the presence of free 131I-TUB-OMOM in the injected mice. Indeed, administration of free 131I-TUB-OMOM to tumor-free mice resulted in rapid blood clearance and predominant accumulation in the colon and ileum content (Fig. 5), and (ii) elevated uptake of 89Zr in comparison with 131I in tumors. This is most probably due to the fact that 131I is released from the tumor after internalization, while 89Zr residuals inside the cell. As such, diverging 89Zr and 131I tumor-uptake levels might be indicative for the level of in vivo ADC internalization (32). At later time points after injection of 131I-TUB-OMOM–89Zr-trastuzumab, diverging levels of 89Zr and 131I were also observed in normal tissues. Whether this is the result of intra- and/or extracellular degradation of the ADC is subject of further investigations.

In vivo therapy studies with a single dose of TUB-OMOM–trastuzumab showed dose-dependent antitumor effects in established N87 tumor-bearing mice. The highest dose, 60 mg/kg, exhibited the same effect as ado-trastuzumab emtansine (15 mg/kg) and a more pronounced effect than trastuzumab alone. No weight loss or other signs of toxicity occurred. Complete eradication of N87 tumors was observed with 30 mg/kg TUB-OMOM–trastuzumab (n = 1), 60 mg/kg TUB-OMOM–trastuzumab (n = 3), and 15 mg/kg ado-trastuzumab emtansine (n = 3). Neither TUB-OMOM–trastuzumab nor ado-trastuzumab emtansine caused antitumor effects in mice bearing the JIMT xenograft line, despite the fact that TUB-OMOM–trastuzumab is targeting JIMT and N87 tumors equally well (see Fig. 4 and Supplementary Fig. S3). The ADC is degraded more rapidly in JIMT tumors than in N87 tumors, resulting in release of 131I from the tumor, and this might be one of the reasons why JIMT tumors show resistance. To get more insight in drug degradation, LC/MS-MS procedures for tubulysin metabolite analysis in tumors are currently being developed.

The use of radiolabeling strategies not only has value for the development and preclinical validation of ADCs as shown herein, but also might be of help during the clinical development of ADCs. The potential of positron emission tomography (PET) imaging of 89Zr-labeled antibodies (i.e., 89Zr-immuno-PET) in mAb development and applications has been shown for several mAbs directed against growth factors and membrane receptors (33, 34), the HER2 receptor included (35). 89Zr-immuno-PET can learn about the ideal mAb dosing for optimal tumor targeting (e.g., saturation of receptors), the uptake in critical normal tissues to anticipate toxicity, and the interpatient variation in pharmacokinetics and tumor targeting. In analogy, 89Zr-immuno-PET might be supportive in the clinical development of tubulysin-antibody conjugates.

In conclusion, herein we show for the first time the potential of tubulysin-antibody conjugates for cancer treatment, thus opening new avenues in ADC development. Nowadays, fully synthetic tubulysins can be produced on large scale and with a broad chemical diversity (5, 6), resulting in higher potency and chemical stability relative to the natural compounds. Some of these synthetic tubulysins derivatives are 10 to 100 times more...
potent in vitro than the TUB-OMOM as used in the present study (unpublished data). Direct coupling of these tubulysin derivatives to lysine residues of mAbs, via the straightforward method reported here, might result in ADCs more potent than TUB-OMOM–trastuzumab and ado-trastuzumab emtansine. In future strategies for TUB-mAb production, conjugation efficiency can easily be monitored by protein mass spectrometry instead of using radioactivity (36, 37).

Disclosure of Potential Conflicts of Interest

P. Lazzaari has ownership interest (including patents) in KemoTech s.r.l. M. Zanda is CEO of KemoTech s.r.l. has ownership interest (including patents) in KemoTech s.r.l. and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

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Development of Novel ADCs: Conjugation of Tubulysin Analogues to Trastuzumab Monitored by Dual Radiolabeling


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