A Novel Platinum(II)-Based Bifunctional ADC Linker Benchmarked Using $^{89}$Zr-Desferal and Auristatin F-Conjugated Trastuzumab

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Abstract

Greater control is desirable in the stochastic conjugation technology used to synthesize antibody–drug conjugates (ADC). We have shown recently that a fluorescent dye can be stably conjugated to a mAb using a bifunctional platinum(II) linker. Here, we describe the general applicability of this novel linker technology for the preparation of stable and efficacious ADCs. The ethylenediamine platinum(II) moiety, herein called Lx, was coordinated to Desferal (DFO) or auristatin F (AF) to provide storable “seminal” products, which were directly conjugated to unmodified mAbs. Conjugation resulted in ADCs with unimpaired mAb-binding characteristics, DAR in the range of 2.5 to 2.7 and approximately 85% payload bound to the Fc region, presumably to histidine residues. To evaluate the in vivo stability of Lx and its effect on pharmacokinetics and tumor targeting of an ADC, Lx-DFO was conjugated to the HER2 mAb trastuzumab, followed by radiolabeling with $^{89}$Zr. Trastuzumab-Lx-DFO-$^{89}$Zr was stable in vivo and exhibited pharmacokinetic and tumor-targeting properties similar to parental trastuzumab. In a xenograft mouse model of gastric cancer (NCI-N87) or an ado-trastuzumab emtansine-resistant breast cancer (JIMT-1), a single dose of trastuzumab-Lx-AF outperformed its maleimide benchmark trastuzumab-Mal-AF and FDA-approved ado-trastuzumab emtansine. Overall, our findings show the potential of the Lx technology as a robust conjugation platform for the preparation of anticancer ADCs.

Cancer Res; 77(2); 257–67. ©2016 AACR.

Introduction

The vast majority of bioconjugation technologies currently rely on the covalent coupling of organic linkers to proteins. Prime examples are linkers that incorporate a reactive ester or a maleimide for the coupling to lysine or cysteine residues, respectively (1). In the area of antibody–drug conjugates (ADC) both linkers have been successfully applied in the clinically approved ADCs Kadcyla (ado-trastuzumab emtansine, T-DN1; ref. 2) and Adcetris (brentuximab vedotin; ref. 3). In Kadcyla, the cross-linking agent succinimidyl 4-((N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is used to position a non-cleavable linker between the toxin DM1 and the monoclonal antibody (mAb) trastuzumab. In Adcetris, the toxin monomethyl auristatin E is coupled to reduced cysteine disulfide bonds of the mAb brentuximab via a maleimide linker containing the cleavable protease-sensitive valine-citrulline (Val-Cit) moiety. Despite the success of these linker technologies, both types of linkers leave room for improving the efficacy and tolerability of ADCs (4).

Besides the mAb and the drug, the linker system is one of the three primary ADC components that determine which cells are targeted, how the drug is released, and by which mechanism of action the cells will be killed (5, 6). The linker system can affect ADC stability and drug release at several levels, as described by Polakis in his recent review (7), and therefore it can be of key importance for efficacy and tolerability of an ADC. First, the drug can be released from the mAb in the circulation, resulting in sequestration of the drug in normal tissues. Second, the mAb itself can be destabilized by drug conjugation, resulting in faster blood clearance of the ADC, and sequestration in catabolic organs such as liver and spleen (8). Third, after cellular uptake of an ADC and subsequent catabolism, the drug can be detached from the ADC and eventually released from the cell (6, 9–11). It can subsequently kill neighboring cells (also called the ‘bystander effect’), but it can also cause increased toxicity, thus making efficacy and tolerability of an ADC less predictable.

Considering the extensive research invested in new conjugation technologies (1, 12–14), it is remarkable that examples of metal-organic linkers for the preparation of ADCs are still scarce (15). Strong metal-protein binding is known for platinum(II) (16)–a property largely responsible for the dose-limiting toxicity of cisplatin (17). On the other side, this property of cis-platinum complexes opens the intriguing possibility of
using these complexes as linkers in bioconjugation reactions. For instance, the ethylenediamine platinum(II) complex has been used for the conjugation of kinase inhibitors to albumin and lysosome for hepatic, renal, and activated endothelial delivery (18, 19).

Recently, we demonstrated that ethylenediamine platinum(II), herein called ‘Lx,’ can be used for development of ADCs by conjugating an NBD-based fluorophore, which is coordinated to Lx via non-conventional functionalities such as N-alkyl amine or N-heterocyclic ligands, to trastuzumab (20). We showed that the aqueous solubility of the resulting charged Lx-payload complexes was greatly improved. Moreover, Lx is expected to coordinate to a unique subset of amino acids in proteins, most notably histidines, cysteines, and methionines (21–25). This offers a valuable alternative to the currently used coupling to lysines or cysteines.

Herein, we report on the use of Lx for coupling of a diagnostic tracer and of a therapeutic drug to native mAbs, and the in vitro and in vivo characterization of the thus obtained ADCs, which notoriously can be challenging, especially with respect to stability analyses in vivo. To this end, we first evaluated Lx for mAb-coupling with 89Zr-labeled Desferal (DFO). 89Zr-DFO has found valuable applications in so-called immuno-PET, which allows the quantitative evaluation of mAb biodistribution in vivo (26–28). In the present study, trastuzumab-Lx-DFO–89Zr was evaluated in vitro for stability and in vivo for tumor targeting in NCI-N87 tumor–bearing mice, along with the lysine-coupled benchmark 89Zr-DFO-trastuzumab (designated as “89Zr-trastuzumab”).

Lx was subsequently used for coupling of a toxin auristatin F (AF) to trastuzumab, and again 89Zr was exploited to compare the in vivo performance of the ADCs. Biodistribution of 89Zr-trastuzumab-Lx-AF was compared with the biodistribution of the labeled parental mAb 89Zr-trastuzumab in NCI-N87 tumor–bearing mice. Finally, trastuzumab-Lx-AF was evaluated for safety and efficacy in NCI-N87 and JIMT-1 bearing mice, along with benchmark ADCs ado-trastuzumab emtansine and trastuzumab-Mal-AF, in which AF is coupled via a maleimide (designated ‘Mal’) conjugation to the mAb, such as it is the case in Adcetris.

Materials and Methods

Cell lines and general materials

Information on general materials can be found in the Supplementary Information Materials and Methods.

Cell lines used were the breast cancer lines MDA-MB-231, JIMT-1, BT-474, and SK-BR-3, the ovarian cancer cell line SK-OV-3, and the gastric cancer cell line NCI-N87. JIMT-1 was obtained from DSMZ on August 25, 2014, after cytogenetic testing by the supplier, and used within 6 months after resuscitation. NCI-N87, MDA-MB-231, BT-474, SK-OV-3, and SK-BR-3 were obtained from the ATCC United Kingdom on August 25, 2014, after cytogenetic testing by the supplier, and used within 6 months after resuscitation. Cytogenetic testing was not repeated by us. However, all cell lines were controlled by us for primary growth characteristics (morphology and growth rate) and HER2 expression before use in experiments. MDA-MB-231 is a HER2-negative cell line; SK-BR-3, BT-474, SK-OV-3, and NCI-N87 are overexpressing HER2; JIMT-1 is developed from tumor cells of a patient with trastuzumab resistance and is HER2-positive (29).

Preparation of Lx-based ADCs and benchmarks; labeling with 89Zr

The synthesis of Lx-DFO, Lx-AF, and Mal-AF can be found in the Supplementary Information section Chemistry.

Conjugation of Lx-DFO and Lx-AF to mAbs

The Lx complexes Lx-DFO and Lx-AF were conjugated to different mAbs, essentially as described previously (20). In a typical example, trastuzumab (71.0 µL, 21 mg/mL) was diluted with tricine buffer (12.3 µL, 200 mmol/L, pH 8.5), and the platinum complex (20 eq. 40.0 µL, 5 mmol/L stock solution) was added. The mixture was incubated at 37°C for 24 hours in a thermomixer at 550 rpm, followed by addition of thiourea (123.3 µL, 20 mmol/L in H2O) and incubation at 37°C for another 30 minutes. The conjugate was purified using an Amicon Ultra-15 centrifugal filter unit (30 kDa MWCO, Merck Millipore) and PBS. Before conjugation, obinutuzumab, ofatumumab, and IgG B12 were buffer-exchanged and concentrated to 21 mg/mL in a tricine buffer (20 mmol/L, pH 8.5) using Amicon Ultra-15 filters. This conjugation method affords ADCs with DARs of 2.5–2.7, that is, the conjugation efficiency is approximately 15%. Recently, we performed a thorough optimization study and increased the conjugation efficiency up to 90% (article in preparation).

Analytical procedures

Analytic procedures related to the analysis of Lx-based conjugates can be found in the Supplementary Information Materials and Methods.

Quality tests and stability of Lx-based ADCs

Trastuzumab-Lx-DFO, 89Zr and 89Zr-trastuzumab were incubated with 1 volume eq. 50% human serum at 37°C. At different time points, radiochemical purity of the conjugates was assessed by iTLC and radio-HPLC, whereas conjugate integrity was analyzed by SDS-PAGE, followed by phosphorimager analysis. In vitro binding characteristics of the ADCs were determined with the Lindmo-binding assay (30).

In vitro cell viability assay

The effects of Lx-AF, Mal-AF, trastuzumab-Lx-AF, IgG B12-Lx-AF, trastuzumab-Mal-AF, ado-trastuzumab emtansine, and trastuzumab incubation on cell viability of the cell lines MDA-MB-231, JIMT-1, BT-474, SK-BR-3, SK-OV-3, and NCI-N87 were determined with the CellTiter-Blue Assay (Promega) essentially according to Cohen and colleagues (31), see Supplementary Information Materials and Methods.

Biodistribution of Lx-based ADCs

Biodistribution studies were performed with trastuzumab-Lx-DFO, 89Zr, 89Zr-trastuzumab, 89Zr-trastuzumab-Mal-AF, and 89Zr-trastuzumab-Lx-AF conjugates. Biodistribution was compared in female nude mice (Hsd athymic nu/nu, 25–32 g; Harlan CPB) bearing NCI-N87 tumors on both sides. All animal experiments were performed according to the Dutch National Institutes of Health principles of laboratory animal care and Dutch national law. Mice were injected intravenously via the retroorbital plexus, 2.0 MBq/100 µg mAb in 100 µL PBS. Blood was collected by tail laceration at 1, 4, 24, 48, 72, and 96 hours p.i. At 72 or 96 hours after injection, the mice were anesthetized, bled, euthanized, and dissected. Blood and organs were weighed, and the amount of...
radioactivity in each sample was measured in a gamma counter (Wallac LKB-CompuGamma 1282; Pharmacia). Uptake of radioactivity was calculated as percentage of injected dose per gram of tissue (%ID/g).

**In vivo safety and therapy studies with Lx-based ADCs**

For safety assessment, 7 groups of 5 mice were given 15, 30, or 60 mg/kg trastuzumab-Lx-AF, trastuzumab-Mal-AF, or PBS as a control by an intravenous bolus injection via the retroorbital plexus. Body weight was measured twice a week and the humane endpoint was reached when weight loss was >10% compared with control mice. The therapeutic effectiveness of trastuzumab-Lx-AF, trastuzumab-Mal-AF, and ado-trastuzumab emtansine was studied in 7 groups of 7–9 mice with established NCI-N87 or JIMT-1 xenographs. The mean tumor size at the start of the study was 140 ± 58 mm³ and 148 ± 57 mm³ for the study with the NCI-N87 and the JIMT-1 tumors, respectively, and was similar for the different treatment groups. In the study with the NCI-N87 tumors, group 1 to 7 received 100 μL of PBS solution (control group), 15 mg/kg trastuzumab, 15 mg/kg ado-trastuzumab emtansine, 15 mg/kg IgG B12-Lx-AF, 15 mg/kg trastuzumab-Mal-AF, 5 mg/kg of trastuzumab-Lx-AF, and 15 mg/kg trastuzumab-Lx-AF, respectively. Ado-trastuzumab emtansine was included as a reference ADC with proven clinical efficacy, IgG B12-Lx-AF as negative control and trastuzumab-Mal-AF as a benchmark conjugate with an established maleimide linker. In the study with the JIMT-1 tumors, group 1–7 received 100 μL of PBS solution, 15 mg/kg trastuzumab, 15 mg/kg ado-trastuzumab emtansine, 15 and 30 mg/kg trastuzumab-Lx-AF, and 15 and 30 mg/kg trastuzumab-Mal-AF, respectively. Body weight and tumor volume were measured twice a week until 125 days after injection. Cures were defined as no outgrowth of regressed individual tumors during the follow-up period.

**Statistical analysis**

All animal experiments were statistically analyzed using the Welch t test for independent samples. Two-sided significance levels were calculated, and a P value <0.05 was considered statistically significant.

**Results**

**Synthesis of the Lx–DFO complex, its conjugation to mAbs, and labeling with ⁸⁹Zr**

The Lx technology is a two-step method for the conjugation of diagnostic or therapeutic payloads to mAbs (Fig. 1A). In the first step, the payload is coordinated to the cisplatin analogue [PtCl₂(en)], followed by conjugation of the Lx-payload complex to a native mAb.

Previously, we have shown that N-nucleophiles are good platinum coordinating groups providing stable N-Pt bonds (20). We choose to first modify the DFO with a succinic acid group followed by the addition of a piperidine coordination group. Coordination to Lx was effected by activation of [PtCl₂(en)] with AgNO₃, followed by reaction with the piperidine-modified DFO-Fc. Purification of the crude product was performed by preparative HPLC to afford the Lx-DFO complex in >95% purity (Supplementary Information section Chemistry, Scheme S1). The complex was stored as a 5 mmol/L solution in 20 mmol/L saline with no decomposition observed by HPLC after storage at 4°C for at least 6 months.

For conjugation, the Lx-DFO complex was incubated with trastuzumab at 37°C for 24 hours. After purification, the mAb-Lx-DFO (Fig. 1B) formation was clearly observed with size exclusion chromatography (SEC), and no aggregate formation became apparent (Fig. 1C). The Lx-DFO:mAb ratio (DAR) was readily determined by SEC-MS to be 2.6 (Fig. 1D). The versatility of the conjugation method was tested by conjugating trastuzumab, obinutuzumab, and ofatumumab with the Lx-DFO complex under identical conjugation conditions. Before conjugation, the mAb solutions were buffer-exchanged to a tricine buffer affording conjugates (99% monomeric) with average DARs between 2.9 and 3.2 (Supplementary Fig. S1). A consistent DAR distribution profile was observed for different mAbs. The percentage of unconjugated antibody was low (1%–5%), whereas conjugates with DARs in the range of 2–4 and 1–5 represented 76%–78% and 94%–96% of the obtained species, respectively.

The preparation of the lysine-coupled DFO-trastuzumab (structure depicted in Fig. 2A) and radiolabeling of DFO-based conjugates with ⁸⁹Zr were carried out according to Verel and colleagues (32). The radiochemical purity of trastuzumab-Lx-DFO-⁸⁹Zr and ⁸⁹Zr-trastuzumab was >99% and the immunoreactive fraction >90% for both conjugates. Moreover, SDS-PAGE and HPLC analyses showed no formation of aggregates.

**Synthesis of the Lx–AF complex and its conjugation to mAbs**

AF was chosen as the cytotoxic payload for evaluation of the performance of Lx in vivo. It has been shown that its carboxylic group can be modified with a non-cleavable spacer without hampering its activity (33, 34).

To allow stable coupling to Lx, AF was modified with a piperidine coordination group (Fig. 2B; Supplementary Information section Chemistry, Scheme S2). The coordination of the piperidine-AF to Lx and the subsequent conjugation to mAbs was similar as described for the DFO-based conjugate. Conjugations of Lx-AF to trastuzumab afforded ADCs that were 99% monomeric with DARs in the range of 2.5 and 2.7.

To evaluate whether Lx alters the therapeutic efficacy of an AF-based ADC, we designed a drug-linker benchmark having a maleimide (Mal) conjugation group instead of an Lx linker (Fig. 2C; Supplementary Information section Chemistry, Scheme S3). The conjugation method afforded trastuzumab-Mal-AF ADCs that were 99% monomeric and had average DARs ranging from 2.0 to 2.3.

**Molecular characterization of Lx-based ADCs**

To assess the position of the Lx-conjugated payload on the antibody, the ⁸⁹Zr-labeled conjugates were reduced with DTT to separate the heavy chain (HC) from the light chain (LC). SDS-PAGE followed by phosphorimager analysis of trastuzumab-Lx-DFO-⁸⁹Zr showed preferential binding to the HC (89% of radioactivity bound to HC, 11% to LC), whereas for the lysine-coupled ⁸⁹Zr-trastuzumab, an HC:LC ratio of 61:39 was found (Fig. 3A). These data were confirmed by SEC-MS analysis (Supplementary Fig. S2A and S2B).

To obtain further insight into the distribution of the Lx-payload on the mAb, trastuzumab-Lx-AF was digested with pepsin or papain in order to separate the F(ab)₂ or Fab fragments, respectively (Fig. 3B; Supplementary Fig. S3). SEC-MS analysis on the pepsin-digested trastuzumab-Lx-AF revealed an average DAR of 0.38 on the F(ab)₂ fragment, which is 15% of the total payload (DAR 2.7). In other words, ca. 85% of the payload was located on the Fc part of the mAb. This "site-restricted
conjugation” was confirmed by SEC-MS analysis of the papain-digested trastuzumab-Lx-AF.

Quality tests and stability of Lx-based ADCs

The in vitro serum stability of the Lx-based ADCs was determined at 37°C in 50% human serum with trastuzumab-Lx-DFO-68Zr (Supplementary Fig. S4A). Radio-TLC, radio-HPLC, and SDS-PAGE followed by phosphorimager analysis showed no release of 68Zr or DFO-68Zr upon incubation for 200 hours, indicating the stability of the Lx linker. Under these conditions, the immunoreactive fraction decreased with 3%–4% after ca. 300 hours. Results of these quality tests were very similar to the lysine-coupled conjugate 89Zr-trastuzumab (Supplementary Fig. S4B).

To assess the in vivo stability and tumor targeting potential of Lx-based ADCs, a biodistribution study was performed in NCI-N87 bearing mice (Fig. 4). Trastuzumab-Lx-DFO-68Zr and 89Zr-trastuzumab were prepared with relatively low DARs of 0.8 and 0.6, respectively, to avoid pharmacokinetic effects. Overall, 89Zr-levels in organs were similar for both conjugates, with the exception of liver (5.4 ± 0.8 and 4.0 ± 0.3 %ID/g for trastuzumab-Lx-DFO-68Zr and 89Zr-trastuzumab, respectively) and blood (3.8 ± 1.7 and 7.1 ± 2.4 %ID/g, respectively). 89Zr-levels in tumors were similar for both conjugates, demonstrating that incorporation of the platinum(II)-linker allowed for stable conjugation and efficient tumor targeting.

In vitro cell viability assay

The antitumor potential of trastuzumab-Lx-AF was measured with the CellTiter-Blue assay and compared with trastuzumab-Mal-AF and ado-trastuzumab emtansine. Moreover, IgG B12-Lx-AF was included as a negative control.

Trastuzumab-Lx-AF (DAR 2.6), trastuzumab-Mal-AF (DAR 2.3), and ado-trastuzumab emtansine (DAR 3.5) showed similar sub-nanomolar potencies in the HER2-positive cell lines NCI-N87, SK-OV-3, SK-BR-3, and BT-474 with IC50s between 10 and 200 pmol/L (Fig. 5A, Supplementary Table S1). On the other hand, the non-binding IgG B12-Lx-AF (DAR 2.5) did not affect cell viability, indicating a targeted delivery of AF for trastuzumab-Lx-AF and...
trastuzumab-Mal-AF. This is supported by the low potencies of the ADCs in the HER2-negative cell line MDA-MB-231 (Fig. 5B). Interestingly, both AF-based ADCs showed sub-nanomolar potencies in the ado-trastuzumab emtansine-resistant cell line JIMT-1. As would be expected, in this cell line the IC50 of ado-trastuzumab emtansine was an order of magnitude higher (Fig. 5C).

To investigate the effect of a premature release of the drug-linkers Lx-AF and Mal-AF in culture medium or blood, the toxicity of these compounds was determined in all tested cell lines as well (Fig. 5D). Before the experiment, Lx-AF and Mal-AF were quenched with thiourea and N-acetyl cysteine, respectively. In all tested cell lines, the potency of the Lx-drug combination was 103 to 104 fold lower compared to its corresponding ADC, whereas for the maleimide-based linker and its corresponding ADC, this difference was only 10 to 100 times.

**Biodistribution of Lx-based ADCs**

The effect of Lx-conjugated AF on mAb pharmacokinetics, tumor targeting, and biodistribution was examined by injecting NCI-N87 bearing mice with ⁸⁹Zr-trastuzumab-Lx-AF (DAR 2.6), ⁸⁹Zr-trastuzumab-Mal-AF (DAR 2.3) or ⁸⁹Zr-trastuzumab (Fig. 6). For all three conjugates, the DFO-to-trastuzumab ratio was chosen to be < 1.0 in order to minimize the possibility of affecting the biodistribution of the AF-based conjugates.

The blood kinetics did not show significant differences: the blood levels were 28.2 ± 2.2, 29.1 ± 1.6, and 29.0 ± 6.8 %ID/g 1 hour p.i. and slowly decreased to 3.2 ± 1.2, 5.1 ± 2.5, and 5.6 ± 2.1 %ID/g 96 hours p.i. for ⁸⁹Zr-trastuzumab-Lx-AF, ⁸⁹Zr-trastuzumab-Mal-AF, and ⁸⁹Zr-trastuzumab, respectively (Fig. 6A). The tumor uptake of the Lx-conjugated ADC (26.1 ± 4.9 %ID/g) was similar to the uptake of the maleimide-conjugated ADC (31.0 ± 6.0 %ID/g) and the unconjugated trastuzumab (29.2 ± 6.8 %ID/g; Fig. 6B). The excellent tumor selectivity of ⁸⁹Zr-trastuzumab-Lx-AF is visualized by PET-CT (Fig. 6C). No significant differences in ⁸⁹Zr-uptake were observed in normal tissues except for the liver, which was higher for ⁸⁹Zr-trastuzumab-Lx-AF (11.6 ± 2.4 %ID/g) than for ⁸⁹Zr-trastuzumab-Mal-AF (7.0 ± 1.4 %ID/g) and ⁸⁹Zr-trastuzumab (5.7 ± 0.8 %ID/g; Fig. 6B).
In vivo safety and therapy studies with an Lx-based ADC

ADC safety was assessed with trastuzumab-Lx-AF (DAR 2.6) and trastuzumab-Mal-AF (DAR 2.3). Nontumor-bearing mice were injected with 15, 30, or 60 mg/kg as an intravenous single bolus injection. The mice treated with 30 mg/kg trastuzumab-Lx-AF showed a weight loss of 4%, whereas the weight loss increased to 10% for the mice treated with 60 mg/kg trastuzumab-Lx-AF. The weight loss of the mice treated with 60 mg/kg trastuzumab-Mal-AF was 4%.

The in vivo efficacy of the trastuzumab-Lx-AF (DAR 2.6) was assessed in NCI-N87 bearing mice (Fig. 7A). PBS, trastuzumab, and IgG B12-Lx-AF (DAR 2.5) were used as controls, while ado-trastuzumab emtansine (DAR 3.5) and trastuzumab-Mal-AF (DAR 2.0) were included as benchmarks. All ADCs were administered as an intravenous single bolus injection at a dose of 15 mg/kg except where stated otherwise in the legend to Fig. 7.

Initially, all groups injected with trastuzumab-based ADCs showed tumor regression, whereas the trastuzumab control group showed only a delay in growth. Although from day 20 on all the tumors of the group treated with 15 mg/kg ado-trastuzumab emtansine started to regrow, the tumors of both groups injected with 15 mg/kg AF-based ADC continued to regress. The tumors of the group treated with 5 mg/kg trastuzumab-Lx-AF showed the same effect as the 15 mg/kg ado-trastuzumab-Lx-AF group did. Differences in tumor growth between groups treated with trastuzumab-Lx-AF and trastuzumab-Mal-AF (both 15 mg/kg) became clearly apparent after 60 days. Whereas the tumors of mice treated with trastuzumab-Mal-AF started to regrow, the tumors of mice treated with trastuzumab-Lx-AF remained constant in volume till the end of the experiment at day 125. Finally, 8 of the 9 mice treated with trastuzumab-Lx-AF survived, as opposed to 2 of the 9 mice treated with trastuzumab-Mal-AF (Supplementary Fig. S5A).

Figure 3.
Determination of Lx-DFO-89Zr in the trastuzumab-Lx-DFO-89Zr conjugate. A, SDS-PAGE followed by phosphorimager analysis of reduced and non-reduced trastuzumab-Lx-DFO-89Zr (lane 1 and 2, respectively) as well as reduced and non-reduced lysine coupled 89Zr-trastuzumab (lane 3 and 4, respectively). B, Payload distribution after DTT reduction or enzymatic digestions.

Figure 4.
Biodistribution of trastuzumab-Lx-DFO-89Zr (DAR 0.8; gray bars) and 89Zr-trastuzumab (DAR 0.6; black bars) 72 hours after injection in NCI-N87 tumor-bearing mice.
Cures, defined as no outgrowth of regressed individual tumors during the follow-up period, were observed for 12 of the 18 tumors (67%, 6 of the 9 mice) in the trastuzumab–lx–AF–treated group at day 125. At that point, all tumors were cured. Whereas around day 20 some tumors in both the trastuzumab-Mal-AF (square open) and Mal-AF (triangle up open) groups regressed completely and no regrowth was observed until the end of the experiment at day 125. At that point, all tumors were cured in both trastuzumab–lx–AF groups (15 and 30 mg/kg). On the other hand, for the groups treated with 15 and 30 mg/kg trastuzumab–Mal–AF, the number of cures was 9 of the 18 tumors (50%, 5 of the 9 mice) and 6 of the 16 tumors (37.5%, 4 of the 8 mice), respectively (Supplementary Fig. S5B). The number of cures in the group treated with ado-trastuzumab emtansine was 4 of the 16 tumors (25%, 2 of the 8 mice) at day 125.

Discussion

Recently, we explored the potential of ethylenediamine platinum(II), which we termed "Lx," as a metal-organic linker in the preparation of ADCs (20). The concept of this novel linker technology is straightforward: in the first step, a diagnostic or therapeutic payload having a suitable coordination group is coordinated to Lx, affording an Lx-payload complex, which we termed a "semi-final product." Because such a "semi-final product" bears a charge, its water solubility increases considerably. In the second step, this complex is conjugated to a mAb in a straightforward procedure: the Lx-payload is mixed with the antibody solution under slightly basic conditions at 37°C for 24 hours, followed by a post-treatment step with thiourea to remove weakly bound complexes. The formed conjugates were found to be stable in PBS, and showed no loss in mAb-binding affinity (20).

In the present study, we further explored the potential of Lx as an ADC linker, aiming to disclose its distinguished properties. Two Lx-payloads were explored to this end: Lx-DFO, containing the 89Zr-binding chelator Desferal (DFO), and Lx-austatin F (Lx-AF). Lx-DFO-89Zr was used to facilitate quantitative characterization of: (i) Lx conjugation properties, (ii) Lx linker stability in vitro and in vivo, and (iii) tumor targeting of Lx-based ADCs in tumor bearing mice, as assessed by ex vivo biodistribution and PET imaging. A similar exploitation of radiochemistry with PET imaging was recently used by us for the characterization of trastuzumab–tubulysin ADCs (31). Lx-AF was used to assess the therapeutic efficacy and safety of Lx-based ADCs in vitro and in vivo. To rank the potential of Lx and Lx-based ADCs, three types of benchmark/control ADCs were included in these studies: (i) IgG B12-Lx-AF, being a non-binding control ADC, (ii) trastuzumab-Mal-AF, in which AF is coupled to the mAb via maleimide conjugation, as it is the case for brentuximab vedotin, and (iii) the FDA approved ADC ado-trastuzumab emtansine, because this is currently the only trastuzumab-based ADC approved by FDA. Moreover, cell lines with different levels of HER2 expression and different sensitivity to trastuzumab were selected.
Conjugation of Lx–DFO and Lx–AF complexes with trastuzumab at 37°C for 24 hours afforded ADCs that were 99% monomeric and had DARs between 2.5 and 2.7. Depending on the amount of Lx-payload in the conjugation reaction, modulation of DAR is possible. The versatility of the conjugation method was demonstrated by replacing trastuzumab for obinutuzumab and ofatumumab, resulting in ADCs with very similar DAR characteristics. The percentage of unconjugated antibody was consistently less than 5%. Moreover, DAR populations in the range of 2–4 and 1–5 represented 76%–78% and 94%–96% of the species, respectively.

The coupling position of the Lx-payload in the mAb was determined by separation of heavy and light chains via reduction. SEC-MS and SDS-PAGE revealed that ca. 90% of the payload were conjugated to the heavy chain. Further investigation with pepsin and papain digestions revealed that ca. 85% were conjugated to the Fc region. This intriguing property of Lx to bind preferentially to the Fc part of the mAb is advantageous as it reduces the chance of affecting the immunoreactivity of the mAb and ultimately the tumor targeting capacity.

Currently, we are setting up strategies to determine the exact binding places and amino acids involved in the Lx conjugation. On the basis of literature (21–25), the probable amino acids for binding are cysteines, methionines, and histidines. However, cysteines are not present in a reduced form in IgGs. Moreover, model studies and density functional theory (DFT) calculations have shown that Pt-methionine bonds are labile and will become broken after the post-treatment with thiourea (38–40). Thus, the thermodynamically favored histidines are most likely to be the binding sites of platinum(II). In order to verify that, we incubated Lx-DFO with N-acetyl-L-histidine amide and N-acetyl-L-methionine amide using our standard conjugation conditions and found that both amino acid derivatives that mimic the corresponding amino acid residues in a mAb coordinated to Lx (Supplementary Fig. S6A–S6C). After post-treatment with thiourea, the methionine ligand was replaced by thiourea, whereas the histidine-Lx complex was intact confirming that histidines are indeed most likely to be the binding sites for Lx. Considering Lx as a histidine conjugation technology, it is remarkable that the majority of the payload is found on the Fc part of trastuzumab, whereas only 54% (14 of 26) of the total histidine population is present in the Fc region. This result indicates that not all histidine residues are equally accessible for the conjugation with Lx. The crystal structure of IgG B12 indeed reveals that more histidine residues can be found at the surface of the Fc region than at the surface of the two Fab regions. Preferential binding of Pt to the Fc part of a mAb was also found by Lasorsa and colleagues (41) who incubated cisplatin with trastuzumab.

The in vitro antitumor potential of trastuzumab-Lx-drug, along with two anti-HER2 benchmark ADCs, was tested on a panel of HER2-overexpressing cell lines. All three conjugates showed IC_{50}S in the range of 20 to 200 pmol/L for the HER2-overexpressing cell lines SK-OV-3, BT-474, NCI-N87, and SK-BR-3. Interestingly,
both AF-based conjugates showed IC50s in the picomolar range in the trastuzumab and ado-trastuzumab emtansine resistant cell line JIMT-1. Lx-AF was $10^{3}$–$10^{4}$ times less toxic compared to trastuzumab-Lx-AF, whereas Mal-AF was only 10 times less toxic than trastuzumab-Mal-AF. Moreover, Lx-AF is $10^{2}$ to $10^{3}$ times less toxic than Mal-AF. We believe that the low toxicity of the Lx complex is due to the positive charge on platinum, which likely impedes passage through the cell membrane. Hereby, we assume that with our current drug-linker design the drug is released intracellularly after lysosomal degradation. The active component that is released in the cell is presumably histidine-Lx-AF. Low toxicity of Lx-drug and Lx-drug derivatives might have safety advantages for laboratory personnel during ADC manufacturing as well as for patients when exposed to Lx-drug metabolites, for example, after lysis of targeted tumor cells.

In vivo serum stability is an important requirement for an ADC linker. Premature release of the drug from the antibody in the bloodstream results in exposure of the free drug to normal organs, leading to unacceptable toxicities. A comparative biodistribution study of trastuzumab-Lx-DFO-69Zr and 69Zr-trastuzumab in NCI-N87 bearing mice revealed similar 69Zr-levels in the tumors and all organs for both conjugates with the exception of liver. This result indicates that the Lx linker technology allows for stable conjugation and efficient tumor targeting. Hydrophobicity, charge on the payload or spacer, and a too high number of coupled payloads can affect the pharmacokinetic and tumor targeting properties of an ADC (8, 42, 43). Therefore, we performed a second biodistribution study with 69Zr-trastuzumab-Lx-AF and 69Zr-trastuzumab-Mal-AF in NCI-N87 bearing mice. The uptake of 69Zr in the tumor and organs, except for the liver, was similar for 69Zr-trastuzumab-Lx-AF, 69Zr-trastuzumab-Mal-AF, and the unconjugated 69Zr-trastuzumab. Both aforementioned biodistribution studies showed a higher liver uptake for the Lx-based ADCs compared with the benchmarks. A possible explanation for this may be the increased isoelectric point of the Lx-based ADC due to the positive charge on the platinum linker (44, 45). Another explanation may be an alteration of FcRn receptor binding, which is known to play an important role in

**Figure 7.** Therapeutic efficacy of trastuzumab-Lx-AF in NCI-N87 bearing mice (A) and JIMT-1 bearing mice (B). PBS (gray star), trastuzumab (pink diamond), IgG B12-Lx-AF (red circle), ado\-trastuzumab emtansine (green triangle down), trastuzumab-Lx-AF 5 mg/kg (gray square), trastuzumab-Lx-AF 15 mg/kg (black square), trastuzumab-Mal-AF 15 mg/kg (blue triangle up), and trastuzumab-Mal-AF 30 mg/kg (blue open triangle up). Note: in B, trastuzumab-Lx-AF 30 mg/kg is not visible due to overlap with trastuzumab-Lx-AF 15 mg/kg.
the recycling of IgGs and extending their half-life in the circulation (46, 47). Histidines play an important role in FcRn receptor binding and because Lx most probably conjugates to histidines it is possible that the Lx conjugation affects binding kinetics to FcRn and reduces the re-circulation of the mAb.

Finally, the efficacy of trastuzumab-Lx-AF was assessed in the NCI-N87 and the ado-trastuzumab emtansine-resistant JIMT-1 cell lines. The Lx-based conjugate outperformed its maleimide benchmark and the FDA approved ado-trastuzumab emtansine in both efficacy studies. These results are surprising considering that no significant differences between trastuzumab-Lx-AF and trastuzumab-Mal-AF were observed in neither in vitro cell viability assays nor biodistribution studies. These data suggest that the in vitro efficacy of the auristatin F-based conjugate is enhanced by inherent favorable properties of Lx. Currently, we believe that this enhancement effect may be a result of longer cellular retention of the drug due to the positive charge on platinum, which remains attached to the auristatin F after lysosomal degradation. Cisdiamino platinum complexes, with cisplatin being the pioneer example, are known to be cytotoxic being able to cross-link DNA, which ultimately triggers apoptosis. However, the proposed catabolite of an Lx-based ADC is histidine-Lx-AF having non-labile ligands. Thus, the Pt-linker itself is unlikely to be able to cross-link the DNA. Currently, we are focussing on internalization/retention of an Lx-based ADC on a cellular level using the radiochemistry described in this paper. Besides elucidating the biology of the Lx linker, we will bring it in to toxicity studies and first-in-human studies in order to obtain further insight into the therapeutic window. This will allow to assess the true value of the Lx linker technology.

In conclusion, herein we describe a novel ADC linker technology that is based on transition-metal coordination chemistry of the ethylenediamine platinum(II) moiety. The conjugation procedure is robust and straightforward and does not require antibody modification. The formed ADCs are stable both in vitro and in vivo, and physicochemical properties of the native mAb are maintained. Moreover, when combined with trastuzumab and auristatin F, Lx has an enhancing effect on the antitumor efficacy.

Disclosure of Potential Conflicts of Interest
P.J.G.M. Steerink is a chief business officer and H.-J. Houthoff is a CEO and CSO and have ownership interest (including patents) in LinXis B.V. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Prof. Dr. Jan Reedijk (University of Leiden), an international expert in platinum chemistry, for critical reading of the article.

Grant Support
This study was funded by LinXis B.V.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 19, 2016; revised November 1, 2016; accepted November 2, 2016; published OnlineFirst November 21, 2016.

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