Hapten-directed spontaneous disulfide shuffling: a universal technology for site-directed covalent coupling of payloads to antibodies

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ABSTRACT Humanized hapten-binding IgGs were designed with an accessible cysteine close to their binding pockets, for specific covalent payload attachment. Individual analyses of known structures of digoxigenin (Dig)-and fluorescein (Fluo) binding antibodies and a new structure of a biotin (Biot)-binder, revealed a “universal” coupling position (52c2) in proximity to binding pockets but without contributing to hapten interactions. Payloads that carry a free thiol are positioned on the antibody and covalently linked to it via disulfides. Covalent coupling is achieved and driven toward complete (95–100%) payload occupancy by spontaneous redox shuffling between antibody and payload. Attachment at the universal position works with different haptens, antibodies, and payloads. Examples are the haptens Fluo, Dig, and Biot combined with various fluorescent or peptidic payloads. Disulfide-bonded covalent antibody-payload complexes do not dissociate in vitro and in vivo. Coupling requires the designed cysteine and matching payload thiol because payload or antibody without the Cys/thiol are not linked (<5% nonspecific coupling). Hapten-mediated positioning is necessary as hapten-thiol-payload is only coupled to antibodies that bind matching haptens. Covalent complexes are more stable in vivo than noncovalent counterparts because digoxigeninylated or biotinylated fluorescent payloads without disulfide-linkage are cleared more rapidly in mice (approximately 50% reduced 48 hour serum levels) compared with their covalently linked counterparts. The coupling technology is applicable to many haptens and hapten binding antibodies (confirmed by automated analyses of the structures of 140 additional hapten binding antibodies) and can be applied to modulate the pharmacokinetics of small compounds or peptides. It is also suitable to link payloads in a reduction-releasable manner to tumor- or tissue-targeting delivery vehicles.—Dengl, S., Hoffmann, E., Grote, M., Wagner, C., Mundigl, O., Georges, G., Thorey, I., Stubenrauch, K.-G., Bujotzek, A., Josel, H.-P., Dziadek, S., Benz, J., Brinkmann, U. Hapten-directed spontaneous disulfide shuffling: a universal technology for site-directed covalent coupling of payloads to antibodies. FASEB J. 29, 1763–1779 (2015). www.fasebj.org

Key Words: targeted delivery • biotin • X-ray structure • protein engineering • pharmacokinetics

Biologically active small molecules and peptides are successfully applied as therapeutics because their rather small size facilitates tissue distribution and access to target cells. Small size, on the other hand, also confers to these entities unfavorable pharmacokinetic parameters: they are rapidly cleared and hence possess a short serum half-life, which in turn limits drug exposure. To overcome these limitations, small molecules or peptides can be coupled to antibodies or proteins (e.g., serum albumin), which by themselves have benign pharmacokinetic properties (1–6). This modulates the pharmacokinetics of small compounds and peptides and prolongs their serum half-life. Coupling of small molecules or peptide derivatives to antibodies that bind to tumor cell surface antigens is also applied to direct potent small molecules to target cells (e.g., in form of tumor-targeting cytotoxic antibody-drug conjugates (7–12).

Various technologies for coupling payloads to antibodies are available. This includes not only nonspecific chemical attachment to lysine or cysteine (7–9), or directed

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coupling to additionally introduced cysteine (THIOMABS) or artificial amino acids (10–12), but also the use of enzymes to couple to specific sequences (13, 14). These technologies link payloads to antibodies in a manner that does not specifically involve antigen binding sites. These coupling reactions are driven either by chemistry determined by substrate-product kinetics or by enzymatic reactions involving forward and backward reactions. Therefore, they are dependent on special coupling conditions and/or pretreatments or on additional proteins (enzymes) to drive the coupling reaction.

An alternative to these coupling reactions is the attachment of payloads via antigen-mediated binding to the variable region, followed by a reaction that links the payload to the antibody. One example is payload-attachment technologies that utilize “half-catalytic” antibodies to bind reactive payloads into binding pockets, upon which the payload reacts with residues (such as lysine) in the pocket (15, 16). This generates covalent payload conjugates that are stably attached to the antibody. These payloads cannot be released except by proteolysis of the antibody binding region.

We have recently devised another approach to couple small compounds and peptides to antibodies and thereby modulate their pharmacokinetics. This technology bases upon haptenylated payloads that are complexes by antibodies in a noncovalent manner (17). In contrast to stable covalent conjugates, noncovalent binding (whose strength is determined by on and off rates of the hapten binder) enables payload release as well as recapture in the circulation. This is of advantage for applications, which require modulation of pharmacokinetics (PK), yet also need free payload for activity.

Although the noncovalent attachment is suitable to significantly prolong the serum half-life, liberation will over time lead to payload elimination because not all payload rebinds to the antibody (17). To address this issue and devise a hapten-based platform that has payloads even more stably attached to antibodies, we designed a disulfide bond between antibody and payload to stabilize the antibody-hapten-payload complex. The objective was to minimize payload loss, yet to keep a functionality to release payload in reducing environments (e.g., after internalization into vesicular compartments). A further objective was to establish a coupling system that (unlike most site-directed chemical or enzymatic coupling systems) is not based upon one defined hapten or coupling reagent or sequence. Because different payloads vary in their compatibility to coupling systems (e.g., restrictions concerning attachment position, size and identity of added entities, biophysical properties), we aimed at developing a universal hapten-based coupling technology that can be applied to a multitude of different hapten, different hapten-binders, and different payloads.

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Materials and Methods

Crystallization and X-ray structure determination of anti-Biot antibody Fab fragments in complex with biocytinamide

Crystals of the antibody Fab fragment (Fab) were grown in 0.8 M succinic acid to a final size of 0.25 × 0.04 × 0.04 mm within 5 days. Biocytinamide (Roche Diagnostics, Penzberg, Germany; in-house production) was dissolved at 100 mM in water. Subsequently, the compound was diluted to 10 mM working concentration in crystallization solution and applied to the crystals in the crystallization droplet. Crystals were washed 3 times with 2 μl of 10 mM compound solution and were finally incubated for 16 hours with biocytinamide at 21°C. Crystals were harvested with 15% glycerol as cryoprotectant and then flash frozen in liquid N2. Diffraction images were collected with a Pilatus 6M detector at a temperature of 100K at the beam line ×105A of the Swiss Light Source (Villigen, Switzerland) and processed with the XDS programs (18) and scaled with SAVERS (BRUKER AXS; Karlsruhe, Germany) yielding data to 2.55 Å resolution. Standard crystallographic programs from the CCP4 software (19) suite were used to solve the structure by molecular replacement with the coordinates of the apo Fab as search model, to calculate the electron density, and to refine the X-ray structure to a resolution of 2.5 Å. The structural models were rebuilt into the electron density using COOT (20). Coordinates were refined with REFMAC5 (21) and with autoBUSTER (Global Phasing Ltd.; https://www.globalphasing.com/buster/).

Structure-based identification of a universal coupling position

To obtain a position that fulfills the rules described in the Results section for all 3 template antibodies, we carried out the following calculations based on the coordinates of the crystallographic structures:

To identify relevant positions that fulfill rule i, we selected residues with a maximum distance of 15 Å from the hapten molecule. This is sufficient to include amino acids in vicinity of the hapten binding pocket at a reasonable distance for reaction with a thiol-containing linker. However, it also selects residues that are located “below” the curvature of the hapten binding pocket (toward the constant domains of the Fab) that would not be reachable by a linker attached to the hapten. We excluded these positions by defining a best fit plane defined by 4 highly conserved antibody residues: the Cα atoms of the cysteine-residues that form the intra-V-domain disulfide bridges (Supplemental Fig. 1). Only residues that lie “above” this plane (in the direction of the paratope) are kept for further processing.

To exclude residues that are directly involved in hapten binding (rule ii), we deselected positions within a distance of 5 Å from the hapten molecule.

To define residues that fulfill rule iii and rule iv, we calculated the relative solvent accessible area for side chain atoms (%SAS) for each residue from steps 1 and 2. %SAS is defined as the area of the amino acid in context of the antibody structure, traced by a by the center of a sphere with the radius of a water molecule (1.4 Å). This area is related to the area for the same amino acid in a Gly-X-Gly tripeptide context with the main chain in an extended conformation (22). As a cutoff for exposed residues, we used values > 60% %SAS. The resulting group of amino acids fulfills rule iii as they are located on the antibody surface and thus should not contribute to intra- and intermolecular interactions of the V-domains, which means that antibody stability should be maintained...
upon mutation. Additionally, these residues fulfill rule iv as the thiol-groups of cysteines in these positions should be fully accessible and reactive.

The residues resulting from steps 1–3 are defined as the “rim region” of the hapten-antibodies.

Criteria for a “universal coupling position (rule v): as a first criterion, we selected residues from steps 1–3 that are found in structurally conserved positions for all 3 template antibodies. This resulted in the identification of 6 positions (according to the Kabat numbering scheme [23]):
- Positions 28 and 31 within complementarity determining region 1 (CDR1) of the heavy chain (HC).
- Positions 53 (or 52b for the Fluo antibody) at the tip of CDR2 of the HC. Despite the difference in Kabat numbering, the identified residues are topologically similar for the 3 anti-hapten antibodies.
- Positions 27 and 30 (or 27c for the Fluo antibody) within CDR1 of the light chain (LC).
- Position 52 within CDR2 of the LC.

**Generation and characterization of hapten binding antibodies**

*Expression and purification of hapten binding antibodies and 52+2 Cys derivatives*

Plasmids that encode HC s or LCs of desired antibodies or antibody derivatives transcribed by a cytomegalovirus promoter were cotransfected into human embryonic kidney 293 suspension cells grown at 37°C in a humidified 8% CO₂ environment in shaker flasks for 7 days after transfection. The encoded antibodies are transiently expressed and secreted in supernatants. Cell culture supernatants containing the antibodies (between 80 and 150 mg/L) are sterile filtered and either stored for up to 15 hours at 4°C for immediate processing (purification), or stored frozen at −80°C and thawed for processing at later time points.

Purification of antibodies from cleared cell culture supernatants was conducted by applying protein A affinity chromatography (MAbSelectSure ProteinA) followed by size-exclusion chromatography (SEC) on a Superdex200 HiLoad 26/60 (both from GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) equilibrated with 20 mM histidine, 140 mM NaCl, at pH 6.0 on an Akta Avant (GE Healthcare) as described previously (24). Protein concentrations were determined by measuring absorbance at 280 nm and product purity was evaluated by analytical SEC and SDS-PAGE to confirm homogeneity of the preparations and absence of aggregates (Fig. 1). Sterile filtered aliquots of the purified antibodies were stored in 20 mM histidine, 140 mM NaCl, at pH 6.0 buffer at −80°C.

**Characterization of hapten binding antibodies and 52+2 Cys derivatives by mass spectrometry and surface plasmon resonance**

The purified antibodies were characterized by mass spectrometry on a NanoElectrospray Q-TOF analyzer after removal of N-glycans by enzymatic treatment with Peptide-N-Glycosidase F (Roche Molecular Biochemicals, Penzberg, Germany). The relative amount of molecule species was thereafter determined by integrating the respective peaks in mass spectra. The results of these analyses are summarized in Table 1 and revealed that antibody derivatives with the 52+2 mutation were quantitatively modified at this position, without any evidence for a free thiol. All antibodies had this position occupied by a cysteine or homocysteine.

To evaluate if the 52+2 Cys mutation influences the binding of the hapten into its binding pockets, affinity measurements were performed. A Biacore T100 or Biacore 3000 instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for surface plasmon resonance experiments with Hapes-buffered saline as running and dilution buffer. Anti-human IgG antibody immobilized via amine coupling on the surface of a CM3 chip was applied to capture the test antibodies as previously described (25). To evaluate the Biot binding antibody, monobiotylated siRNA was applied as ligand, which was injected at increasing concentrations (0.4–300 nM). Regeneration was with 0.85% H₃PO₄ for 60 seconds at 5 μl/min followed by 5 mM NaOH for 60 seconds at 5 μl/min to remove any noncovalently bound protein after each binding cycle. Test samples were diluted in Hapes-buffered saline (10 mM Hapes, pH 7.4, 150 mM NaCl, 0.005% surfactant P20) and injected at a flow rate of 5 μl/min. The contact time (association phase) was 3 minutes for the antibodies at a concentration between 1 and 5 nM and the dissociation time (washing with running buffer) 5 minutes for each molecule at a flow rate of 30 μl/min. All interactions were performed at 25°C and signals were detected at a rate of 1 signal per second. The derived curves were fitted to a 1 Langmuir binding model using the biacore evaluation software. The comparison of the binding affinities of the 52+2 Cys variant with those of the parent antibodies demonstrated that a Dig or Biot payload binds to its cognate mutated antibody derivative in the same manner as to the parent antibodies. Thus, the binding functionalities of the 52+2 derivatives are retained. In the same manner, surface plasmon resonance analyses were performed to analyze if the introduction of a thiol into the hapten-payload module influences hapten positioning. The results of these analyses revealed that the thiol containing Dig- or Biot-CysCy5 binds to its cognate parent antibody (which cannot form a disulfide with the payload) in the same manner as the control payloads.

**Generation of haptenylated payloads**

**Dig-Cy5**

The generation of Dig-Cy5 has been previously described (24). In short, DIG-carboxymethyl-N-hydroxysuccinimide (NHS) ester (DE 3836656) was transformed with mono-boc-ethylenediamine. Afterward, Boc was removed and the released amine was allowed to react with Cy5-NHS ester (GE Healthcare). Dig-Cy5 was purified by HPLC (RP 18), eluted over 60 minutes with a 0–100% gradient of H₂O containing 0.1% trifluoroacetic acid (TFA) (eluent A) and acetonitrile containing 0.1% TFA (eluent B). The molecular weight of Cy5 is 791.99 Da and that of the resulting Cy5-Dig is 1167.55 Da. Until the point of complexation to the antibody, Dig-Cy5 was stored in aliquots in PBS at −20°C.

**Dig-Cys-Cy5**

In an Erlenmeyer flask, 1,2-diamino-propane trityl resin (250 mg, 0.225 mmol, loading 0.9 mmol/g) was swelled with dime-thoxyformamide (DMF) (5 ml) for 30 minutes. Subsequently, a solution of FMOC-Cys(Trr)-OH (395 mg, 0.675 mmol) in DMF (2 ml) and a solution of HATU [N-2(7-azabenzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium-hexafluorophosphate] (433 mg, 1.2375 mmol), and 1-hydroxy-7-azabenzotriazol (HOAt) (164 mg, 1.2375 mmol) in DMF (8 ml) were added to the resin. To this suspension was added diisopropylethylamin (DIPEA) (385 μl, 2.25 mmol) and the mixture was swirled for 16 hours at ambient temperature, filtered, and washed repeatedly with DMF. After the coupling step, unreacted amino groups were capped by treatment with a mixture of acetic anhydride (20%) in DMF followed by a washing step with DMF. Removal of the N-terminal FMoc group was accomplished by treatment of the resin with piperidine (20%) in DMF. Afterward, the resin was washed thoroughly with DMF and isopropanol, and again with DMF, and was then treated with a solution of Cy5-Mono NHS ester (25 mg,

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0.0316 mmol) in 1% DIPEA in DMF (10 ml) for 16 h. After filtration and washing with DMF, the resin was treated with a mixture of trifluoroacetic acid, water, and tri-isopropylsilane (9 ml:9 ml:1 ml) for 3 hours. The cleavage solution was filtered, concentrated under reduced pressure, and the resulting solid was redissolved in water and lyophilized. Purification of the intermediate was accomplished by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Chromolith prep RP-18e column, 100 x 25 mm; Merck, Darmstadt, Germany) resulting in a blue solid after lyophilization. Analytical HPLC: retention time (tR) = 6.2 min (Merck Chromolith Performance RP-18e, 100 x 4.6 mm, water + 0.1% TFA → acetonitrile/water + 0.1% TFA 80:20, 25 minutes. Subsequently, a portion of this intermediate (6.5 mg, 7.9 μmol) was dissolved in DMF (1 ml) and a solution of Dig-Amcap-OSu (5.2 mg, 7.9 μmol) in DMF (1 ml) and 1% triethylamine in DMF (2 ml) were added and the mixture was tumbled for 16 hours. The solution was concentrated afterward, and the target compound was purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 x 25 mm), for a yield of 3 mg. Analytical HPLC: tR = 8.7 minutes (Merck Chromolith Performance RP-18e, 100 x 4.6 mm, water + 0.1% TFA → acetonitrile/water + 0.1% TFA 80:20, 25 minutes. Electrospray ionization-mass spectrometry (ESI-MS) (positive ion mode): m/z calculated for [M]: 1360.0; found: 1360.7 [M+H]1+.

**TABLE 1.** Mass spectrometry analyses of antibody payload complexes reveal the content of covalently linked payload within the antibody-payload complexes

| Antibody  | Payload          | IgG + 2 payloads | IgG + 1 payload | IgG without payload |
|-----------|------------------|------------------|-----------------|--------------------|
| <Biot> Cys52b | Bio-Cys-Cy5      | >95%             | –               | –                  |
| <Biot> Cys52b | Bio-Cys-PYY      | >95%             | –               | –                  |
| <Dig> Cys52b | Dig-Cys-PYY      | >80%             | <20%            | –                  |
| <Fluo> Cys52b | Fluo-Cys-PYY     | >95%             | –               | –                  |

See Supplemental Data for details.
A FMOc-2-(3-[2-(2-amino-ethoxy)-ethoxy]-ethoxy)-propionyl-Cys-diamino-propane (= FMOc-PEG-Cys) modified resin was synthesized according to standard peptide synthesis procedures. Removal of the N-terminal Fmoc group was accomplished by treatment of the resin (34 mg) with 4 x 5 ml pipеридине (20%) in DMF. Afterward, the resin was washed thoroughly with DMF and isopropanol, and tried. Resin (28 mg) was then treated with a solution of Cys-Mono NHS-ester (14 mg, 0.0175 mmol) in 1% DIPEA in DMF (5 ml) for 40 hours. The resin was filtered and washed with DMF and isopropanol. The resin was treated with 10 ml of a mixture of trifluoroacetic acid, water, and triisopropylsilane (9 ml:9 ml:1 ml) for 2.5 hours. The cleavage solution was filtered, concentrated under reduced pressure, and the resulting solid was redissolved in water and lyophilized. Purification of the intermediate was accomplished by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (C8-5 μm column, 250 x 20 mm; Nucleosil; Bischoff, Leonburg, Germany) resulting in a blue solid after lyophilization, for a yield of 1 mg. Analytical HPLC: tR = 7.38 minutes (Nucleodur, 150 x 3 mm, C18-100-3 μm, water + 0.1% TFA → 95% acetonitrile + 0.1% TFA, 15 minutes). Subsequently, this intermediate (1 mg, 0.001 mmol) was dissolved in DMF (1.5 ml) and 3 μl triethylamine and Dig-Anacap-OSu (1.89 mg, 0.0028 mmol) were added, and the mixture was stirred for 16 hours. The reaction mixture was concentrated afterward, and the target compound was purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 1% TFA (Hyperlite Gold, C18-8 μm column, 100 x 10 mm; Thermo Fisher Scientific, Waltham, MA, USA), for a yield of 1.46 mg. Analytical HPLC: tR = 9.05 min (Nucleodur, 150 x 3 mm, C18-100-3 μm, water + 0.1% TFA → 95% acetonitrile + 0.1% TFA, 15 minutes). ESI-MS (positive ion mode): m/z calculated for [M]: 1561.74; found: 1562.4 [M+H]+.

**Biotin-Ser-Cy5**

This molecule was synthesized in 4 steps. Step 1 generated Biotin-O2Oc-Cys-O2Oc-DADOO-NH₂: On an Obis-(aminoethyl)ethyl methacrylate glycol trityl resin (352 mg, 0.25 mmol, loading 0.71 mmol/g; Novabiochem) Fmoc-O2Oc-OH, Fmoc-Cys(Trr)-OH, Fmoc-O2Oc-OH (all Iris Biotech), and DMTr-D-Biotin (Roche) were coupled consecutively. Peptide synthesis was performed according to established protocols (FastMoc 0.25 mmol) in an automated Applied Biosystems ABI 433A peptide synthesizer using Fmoc chemistry (as described for SEQ ID NO: 180). After synthesis, the resin was washed thoroughly with DMF, methanol, dichloromethane, and dried under vacuum. Then, the resin was placed into an Erlenmeyer flask and treated with a mixture of trifluoroacetic acid, water, and triisopropylsilane (9.5 ml:250 μl:250 μl) for 2 hours at room temperature. The cleavage solution was filtered and the peptide was precipitated by addition of cold (0°C) diisopropyl ether (100 μl) to furnish a colorless solid, which was repeatedly washed with diisopropyl ether. The crude product was redissolved in water, lyophilized, and subsequently purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 x 25 mm) resulting in a colorless solid after lyophilization for a yield of 79 mg (41%). Analytical HPLC: tR = 5.5 minutes (Merck Chromolith Performance RP-18e, 100 x 3 mm, water + 0.1% TFA → acetoni thetritol + 0.1% TFA, 15 minutes). ESI-MS (positive ion mode): m/z calculated for [M]: 767.9; found: 768.44 [M+H]+; 384.8 [M+H]+.

Step 2, Biotin-O2Oc-Cys(TNB)-O2Oc-DADOO-NH₂: The peptide (30 mg, 39 μmol) was dissolved in 100 mM potassium phosphate buffer, pH 7.5 (4 ml) and 5.5'-dithiothreitol (2-minitrobenzoic acid) (77 mg, 195 μmol) was added. The mixture was stirred for 30 minutes at room temperature and subsequently purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 x 25 mm) resulting in a colorless solid after lyophilization for a yield of 31 mg (83%). Analytical HPLC: tR = 5.4 minutes (Merck Chromolith Performance RP-18e, 100 x 3 mm, water + 0.025% TFA → acetoni thetritol + 0.025% TFA, 15 minutes. ESI-MS (positive ion mode): m/z calculated for [M]: 965.1; found: 965.4 [M+H]+; 483.3 [M+H]+.

Step 3, Biotin-O2Oc-Cys(TNB)-O2Oc-DADOO-Cy5: The TNB protected peptide (9.9 mg, 10.3 μmol) was dissolved in 200 mM potassium phosphate buffer, pH 7.5 (1026 μl). Cy5-Mono NHS-ester (6.5 mg, 8.2 μmol; GE Healthcare) was dissolved in water (1026 μl) and added to the peptide solution. The reaction solution was stirred for 2 hours at room temperature and was subsequently purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 x 25 mm) resulting in a blue solid after lyophilization for a yield of 10 mg (80%). Analytical HPLC: tR = 7.2 minutes (Merck Chromolith Performance RP-18e, 100 x 3 mm, water + 0.025% TFA → acetoni thetritol + 0.025% TFA, 15 minutes. ESI-MS (positive ion mode): m/z calculated for [M]: 1603.9; found: 1604.9 [M+H]+; 803.1 [M+H]+.

Step 4, Biotin-O2Oc-Cys-O2Oc-DADOO-Cy5: The TNB protected and dye labeled peptide (10 mg, 6.1 μmol) was dissolved in water (789 μl) and added to the peptide solution. The reaction solution was stirred for 2 hours at room temperature and was subsequently purified by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 x 25 mm) resulting in a blue solid after lyophilization for a yield of 6 mg (58%). Analytical HPLC: tR = 6.1 minutes (Merck Chromolith Performance RP-18e, 100 x 3 mm, water + 0.025% TFA → acetoni thetritol + 0.025% TFA, 15 minutes. ESI-MS (positive ion mode): m/z calculated for [M]: 1390.7; found: 1391.2 [M+H]+.
dissolved in a mixture of 200 mM potassium phosphate buffer, pH 7.5 (1522 μl) and water (1218 μl); 100 mM tris(2-carboxyethyl)phosphine hydrochloride solution (304 μl) was added and the reaction mixture was stirred for 30 minutes at room temperature. Purification was performed by preparative reversed phase HPLC using an acetonitrile/water gradient containing 0.1% TFA (Merck Chromolith prep RP-18e column, 100 × 25 mm) resulting in a blue solid after lyophilization for a yield of 7.6 mg (86%). Analytical HPLC: tR = 6.4 minutes (Merck Chromolith Performance RP-18e, 100 × 3 mm, water + 0.2% TFA → acetonitrile/water + 0.2% TFA 80:20, 25 minutes. ES-MS (positive ion mode): m/z calculated for [M]+: 1406.8; found: 1406.8 [M-H]+; 704.0 [M+2H]+.

Haptenylated PYY-derived peptides

The peptide tyrosine tyrosine or pancreatic peptide YY short PYY (3–36) analog (17) is coupled to hapten via the amino acid residue lysine in position 2. PYY derivatives coupled with or without cysteine in its linker to haptons were generated by first synthesizing a PYY precursor molecule (moPYY) and subsequently coupling it to the desired linker-hapten.

moPYY precursor

The PYY (3–36)-polypeptide derivative ( termed moPYY) was obtained by automated solid-phase synthesis of the resin-bound peptide sequence Ac-IK(Mmt)-Pqa-R(Pbf)H(Trt)Y(tBu)LN(Trt)W(Boc)VT(tBu)R(Pbf)Q(Trt)-MeArg(Mtr)-Y(tBu)-TentaGel S RAM resin. Peptide synthesis was performed according in a Multiple Synthesizer SYRO I (MultiSynTech GmbH, Witten, Germany) with vortex stirring system using Fmoc chemistry. Using a TentaGel RAM resin (loading: 0.25 mmol/g; Rapp Polymers, Thueringen, Germany), the peptide sequence was assembled in iterative cycles by sequential coupling of the standard protocol, double coupling of Fmoc-PEG3-OH (by means of the standard protocol), double coupling of Fmoc-Cys(Trt)-OH (by means of the standard protocol), double coupling of Fmoc-PEG2-OH with 57.8 mg (3 equiv.) Fmoc-8-amino-dioxaoctanoic acid (PEG2 spacer), 48.2 mg (3 equiv.) TBTU and 33.3 mg (6 equiv.) NMM in 1.2 ml DMF, 2 × 30 minutes and biotinylation with a solution of 48.9 mg Biot (4 equiv.), 64.2 mg TBTU (4 equiv.) and 44.4 μM NMM (8 equiv.) in 1.2 ml NMP, (preactivation 5 minutes), single coupling 2 hours. For Biot-ser-moPYY, the linker cysteine was replaced by a serine, applying otherwise identical synthesis procedures. Cleavage from the resin, purification, and analysis was performed as described (m/z calculated for C134H203N39O32S2 = 2936.5; found: 2937.8). For Biot-ser-moPYY, the linker cysteine was replaced by a serine, applying otherwise identical synthesis procedures.

Fluo-Cys-moPYY

Ac-IK(PEG3-Cys-4-Abu-5-Fluo)-Pqa-RHYNLNVTRQ-MeArg-Y-NH2/Ac-PYY(PEG3-Cys-4-Abu-5-Fluo): Starting with the precursor Ac-IK-Pqa-R(Pbf)H(Trt)Y(tBu)LN(Trt)W(Boc)VT(But)-R(Pbf)Q(Trt)-MeArg(Mtr)-Y(tBu)-TentaGel S RAM resin, the peptide synthesis was continued analogously to Bio-Cys-moPYY except that 54.2 mg 5-carboxyfluorescein, 33.1 mg hydroxybencozine (HOBt) and 35.6 μl diisopropylcarboxylamide (DIC) in DMF was added instead of Biot (m/z calculated for C143H195N37O34S = 3008.44; found: 2968.2). Ac-IK(PEG3-Cys-PEG2-Fluo)-Pqa-RHYNLNVTRQ-MeArg-Y-NH2/Ac-PYY(PEG3-Cys-PEG2-Fluo): Starting with the precursor Ac-IK-Pqa-R(Pbf)H(Trt)Y(tBu)LN(Trt)W(Boc)VT(But)-R(Pbf)Q(Trt)-MeArg(Mtr)-Y(tBu)-TentaGel S RAM resin, the peptide sequence was continued as described (m/z calculated for C131H197N39O30S2 = 2862.4; found: 2937.8).

Generation and characterization of antibody-payload complexes

Antibody-payload complexes

Noncovalent complexes of antibodies and payloads were generated by mixing a 10 μg/ml antibody solution in 20 mM histidine, 140 mM NaCl, pH 6.0 with 2.5 molar equivalents of the respective protocol. Following double couplings of Fmoc-Cys(Trt)-OH and Fmoc-Ba-Ala-OH performed automatically in the SYRO 1 synthesizer by means of the standard protocol, a solution of biotin-OH in NMP prepared from 48.9 mg Biot (4 equiv.), 64.2 mg TBTU (4 equiv.) and 44.4 μM NMM (8 equiv.) in 1.2 ml NMP, (preactivation 5 minutes) was added manually and stirred at room temperature. After 2 hours, the resin was washed with DMF, EtOH, methyl tert-butyl ether (MTBE), and dried. For Biot-ser-moPYY, the linker cysteine was replaced by a serine, applying otherwise identical synthesis procedures. Final cleavage was performed as described above. The crude product was purified by preparative reversed phase HPLC as previously described (17) giving a colorless solid (m/z calculated for C131H197N37O34S = 3008.44; found: 2968.2).
payload solubilized in the same buffer or in 100% DMF (anhydrous; Solulink, San Diego, CA, USA), dependent on the properties of the compound. The solutions were incubated for 1 hour at 25°C while slowly agitated by shaking. For payloads >3000 Da, the charging ratio of antibody to payload was measured by SEC-MALLS using a Dionex Ultimate 3000 HPLC system together with HELEOS II/QELS- and Optilab rEX detectors (Wyatt, Santa Barbara, CA, USA). Sample (50 µg) was applied to a Superdex 200 10/300 GL column (GE Healthcare) using PBS pH 7.4 as liquid phase at a flow rate 0.5 ml/min. For haptenylated compounds carrying fluorescent dyes, the antibody/payload ratio was calculated with the formula \( \frac{A_{\text{dye}}}{A_{\text{payload}}} \) protein concentration (M), where \( A_{\text{dye}} \) is the absorption of the sample at the absorption maximum wavelength of the dye (650 nm for Cy5) and \( A_{\text{payload}} \) is the molar extinction coefficient of the fluorescent dye (250,000 M\(^{-1}\)cm\(^{-1}\) for Cy5). The protein concentration was measured at 280 nm. Because absorption of Cy5 at 280 nm was negligible, no correction factor was applied.

Hapten-positioned covalent coupling

For the generation of covalently linked antibody-payload combinations, 52 Cys mutated antibodies were dialyzed into complexation buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA) using Slide-A-Lyzer cassettes (30K MWCO; Thermo Fisher Scientific). Dialysis was carried out in 2 steps for 3–5 hours and overnight at 4°C. The protein concentration of the antibody solution was adjusted to 5–10 mg/ml. The respective payload was solubilized in a suitable buffer (usually complexation buffer) at a concentration of 10 mg/ml. Payload that were not soluble in aqueous buffer were solubilized in 100% DMF (anhydrous; Solulink). Immediately after solubilization, the solutions were mixed at a molar ratio of 1:2.5 antibody/payload while slowly agitated by shaking or stirring with a polytetrafluoroethylene (PTFE) stirrer bar. The mixture was incubated for 1 hour at 25°C. Subsequently, the buffer was exchanged to 20 mM histidine, 140 mM NaCl pH 6.0 either by dialysis (Slide-A-Lyzer cassettes, 90K MWCO; Thermo Fisher Scientific) or by size exclusion chromatography (Superdex 200; GE Healthcare) depending on the scale of the reaction. Characterization of the samples was carried out by fluorescent SDS-PAGE or Western blot and mass spectrometry analysis.

Analysis of payload coupling by SDS-PAGE and subsequent fluorescence imaging or Western blot detection, and by mass spectrometry analyses

For SDS-PAGE, 3–5 µg samples from conjugation reactions were mixed with 4× NuPAGE LDS Sample buffer (Invitrogen, Carlsbad, CA, USA) and incubated at 95°C for 5 minutes. Reduced samples were produced by adding NuPAGE sample reducing agent (10×, Invitrogen). Proteins and peptides were separated on a NuPAGE 4–12% Bis-Tris acrylamide gel in an Xcell SureLock electrophoresis chamber (Invitrogen). Haptenylated compounds conjugated with fluorescent Cy5 or compounds with Fluo as hapten were detected with a Lumi-Imager F1 (Roche Molecular Biochemicals). After fluorescence detection, the gels were stained with SimplyBlue SafeStain (Invitrogen) to reveal the whole protein content of each sample. Mass spectrometry analyses of antibody-payload combinations were performed on a NanoElectrospray Q-TOF analyzer after removal of N-glycans by enzymatic treatment with peptide-N-Glycosidase F (Roche Molecular Biochemicals). The relative amount of molecule species was thereafter determined by integrating the respective peaks in mass spectra.

Pharmacokinetics of antibody-payload complexes

Experimental study protocols were reviewed and approved by the local government, and all studies were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved animal facility. To analyze the serum half-life of antibody-hapten conjugates and antibody-hapten complexes in mice, 2,14 mg (14.4 nmol) of Biot antibody complexed to Biot-Ser-Cy5 and Biot 52 Cys conjugated with Biot-Cys-Cy5 were applied to 6 female NMRI mice each. The volume of each dose was 0.2 ml, and administration of the compounds was carried out intravenously into the tail. Blood was collected via the retrobulbar venous plexus. Blood samples were taken from 2 groups of 3 mice for each compound after 0.08, 4, and 48 hours for the first group and after 0.08, 24, and 72 hours for the second group. For each time point, ~0.1 ml of blood was transferred to hemocytor capillary into microtubes 500 Z-Gel (Sarstedt, Nürnberg, Germany). At least 50 µl of serum were obtained from blood after 1 hour at room temperature by centrifugation (9300 g) at 4°C for 3 minutes. Samples were frozen directly after centrifugation and stored at –80°C until analysis. Cy5-related fluorescence in the serum samples was measured with a Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA, USA) with an excitation wavelength of 640 nm and an emission wavelength of 667 nm. The mean of the fluorescence signal at 0.08 hour was set to 100% for each compound. The decrease of Cy5 serum levels was calculated as the relative fluorescence at the respective time points. To determine the amount of human IgG antibody in the serum at the given time points, human IgG antibodies in serum samples were captured on a solid phase (Maxisorp microtiter plate, NUNC4mmumo) coated with an anti-human κ-chain monoclonal IgG antibody. Mouse serum (100 µl) was added in a dilution of 1:100 and 1:1000. Wells were washed 3× with 300 µl 1× PBS. Detection of human IgG antibodies was carried out by first adding 100 µl of anti-human CH1-domain IgG digoxigeninylated at the C terminus at 0.25 µg/ml. After washing 3 times with 300 µl of 1× PBS, 100 µl anti-Dig Fab conjugated to horseradish peroxidase (HRP) was added at a concentration of 25 µU/ml. Biomimicrin signals were generated by addition of the HRP-substrate 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). IgG concentrations were calculated from standard curves. The mean IgG concentration of the \( t=0.08 \) hour samples was set to 100% and the decrease of antibody serum levels was calculated as the relative concentration at the respective time points.

RESULTS

Hapten binding antibodies

Recombinant full-length IgGs that bind the hapten Dig, Fluo, or Biot were used as template molecules for antibody engineering. The Dig binding antibody is a humanized IgG with low nanomolar affinity (24). Its structure in complex with Dig has previously been elucidated (PDB code: 3RA7; 18, Fig. 2A). The anti-Fluo antibody 4M5.3 is a high-affinity derivative of the murine antibody 4-4-20 (25) that was generated by yeast surface display (26) and converted to a human IgG format for this study. The structure of this antibody complexed with Fluo (PDB code: 1X9Q; 27) is shown in Fig. 2B. The Biot binding antibody is a humanized IgG of murine hybridoma origin, which has not been described before. It binds with low nanomolar affinity to Biot derivatives that are coupled to a payload at its carboxyl group. This antibody binds only to linker-coupled Biot derivatives but not to unmodified Biot that contains a carboxyl group. To elucidate the reason for this specificity and to generate the Biot-antibody complex structure for antibody engineering (in concert with the anti-Dig and anti-Fluo structures), we solved an X-ray structure at a resolution

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of 2.45 Å of biocytinamide bound by Fab of the murine anti-Biot antibody (see Materials and Methods; PDB code: 4S1D). It reveals that Biot binds into a pocket that is formed by residues from HC CDRs 1 and 3, and all 3 CDRs of the LC (Fig. 2C). Biot is coordinated by hydrogen bonds to S34 (Kabat positions are applied throughout this article) (23) of LC CDR1 and to S50 of LC CDR2 via the carbonyl group and/or the nitrogens of the ureido moiety. Additional hydrogen bonds are found between the carbonyl group of the Biot amide bond and HC T32 as well as to the backbone nitrogen of HC F33. The terminal amine of biocytinamide is hydrogen-bonded to HC N29. Hydrophobic contacts are provided by the HC CDR3 residues W95 and W100b and the LC Y32 (CDR1) and F91 (CDR3) that form a cage-like structure around Biot. An additional important hydrophobic interaction can be found between F33 of the LC CDR1 that stacks with the Biot amide-bond. Nondervatized Biot carries a negatively charged carboxyl group at the position of the biocytinamide amide bond. The binding pocket surrounding this group is negatively charged (mainly due to D31 of HC CDR1 and D52 of HC CDR2; Fig. 2D), which is shown by calculation of surface electrostatics using the Fab structure without biocytinamide. Thus, electrostatic repulsion between the carboxyl-group of “free” Biot and the negatively charged wall of the binding pocket explains the selectivity for coupled Biot-derivatives without a carboxyl group in the relevant position.

A universal position for covalent disulfide coupling of hapten-positioned payloads

A comparison of the structures of the 3 hapten binding antibodies revealed as commonality a deep hapten binding pocket or cleft, but otherwise a rather different composition of the binding sites for each of the different haptons: the Dig binding pocket is predominantly composed of large aromatic amino acids, which are part of the HC CDRs (Y56 and Y58 in HC CDR2, Y100e and Y100f in HC CDR3). The binding pocket of the anti-Fluo antibody is also dominated by aromatic residues contributing hydrophobic interactions with Fluo. However, the residues are located at positions that are different from the Dig binder (W33 in CDR1, Y53 in CDR2, Y97 in CDR3 of the HC and Y32 in CDR1, W96 in CDR3 of the LC). As described above, the Biot binding pocket again shows differences from the Dig and the Fluo binders. A common feature of all 3 antibodies is that the hapten binds to a pocket or cleft that is surrounded by a “rim region” of residues without major contribution to hapten binding. This is shown exemplary for
the Dig binding antibody in Fig. 3A. The linker connecting the hapten with payloads always extends out of the pocket and thereby passes the rim region. Based upon the structures of the anti-hapten antibodies described above, we identified a position for introduction of a cysteine mutation, which shall enable hapten-positioned covalent coupling of thiol-containing hapten payloads. This position shall enable coupling to each modified antibody, independent of the hapten that is applied for payload positioning. The design for generation of these antibody derivatives included placement of the additional cysteine in a manner that fulfills the following rules:

i. The Cys must be located in proximity to the binding pocket to exploit the hapten positioning effects for site-directed covalent payload coupling.

ii. The Cys mutation must not interfere with binding of different anti-hapten antibodies to allow hapten-mediated payload positioning.

iii. The Cys must be placed at a location that does not interfere with structural integrity of the V-region, to minimize local disturbances of the antibody framework structure and maintain stability.

iv. The thiol group of the Cys must be directed at the solvent exposed surface of the antibody to be accessible to its coupling counterpart on the payload.

v. The location of the Cys in the rim region must be conserved in different hapten binding antibodies, to generate a universally applicable positioning-coupling system.

Calculations based on the crystal structures (Supplemental Data) revealed the design requirements to be fulfilled by 6 positions. Figure 3B shows the location of these positions in the rim region of the anti-Dig antibody. Three of these are in the LC (VL27, VL30, VL52) and the other 3 (28, 31, 53) in the rim region of . From these initial 6 candidates, we focused on the 3 HC positions because LC shuffling is frequently applied (e.g., for maturation purposes). Having the rim-mutation in the HC does not interfere with subsequent LC shuffling or exchange approaches. From the 3 HC positions, we chose 53 for further analyses. The numbering ID of this “noncontact” position depends on the length of CDR-H2, being defined as the second amino acid following H52. It is defined as H54 for antibodies without extended CDR H2, H53 for

Figure 3. VH- and VL-domains are shown in dark and light colors, respectively. A) “Cut” along the V-region of the Dig-binder demonstrates the binding pocket and the rim region (yellow) that surrounds it. B) Six positions in the rim region fulfill the design parameters, 3 in VH and 3 in VL (exemplarily shown for anti-Dig). C) VH 53 in the rim region is in proximity to the binding pocket yet does not contribute to hapten binding or barrel structure. VH 53 (gly, yellow) can be replaced by a cysteine. D) VH 52b (K) in the rim region of the Fluo binding antibody (equivalent in position to 52+2) is shown in yellow. E) 53 (A) in the rim region of the Biot binding antibody (yellow). F) Model of the Dig-antibody-Dig-Cys-Cy5 conjugate. The linker-C53–conjugated structure was manually built followed by a minimization step (CHARMM force-field, conjugate gradient minimization, Discovery Studio 3.5; Biovia, San Diego, CA, USA).
antibodies that have CDR H2 extended by 1 amino acid (e.g., <Dig>), and H52b for antibodies with longer extensions of CDR H2. The topology of these amino acids is similar in different CDR H2 types, even though their actual Kabat positions differ. To accommodate all 3 possibilities, we define this position as “52^2.” This position not only matched all design rules for Cys placement, but it is also, compared with the other positions (H28, H31) in the primary sequence, more distant to the conserved cysteine 22, which is part of the Cys22-Cys92 intradomain disulfide. Thus, compared with the more proximal positions H28 and H31, mutating 52^2 may pose a reduced risk of disulfide scrambling during protein synthesis and hence non-functional protein during antibody production. Figure 3C shows exemplarily for the anti-Dig V-region that the 52^2 Cys mutation in CDR2 locates outward facing on the rim region of the antibody. It disturbs neither the antigen binding site nor the structural integrity of the V region, yet is still in proximity to the binding pocket. The position and orientation of 52^2 on the rim region of the binding pocket is also retained in the direct and Biot binding antibodies (Fig. 3D, E). All parent antibodies and engineered derivatives were transiently expressed as secreted proteins in human embryonic kidney 293 cells and purified to homogeneity from cell cultures supernatants by protein A and size exclusion chromatography (see Materials and Methods). To confirm that the 52^2 mutation does not affect hapten binding, surface plasmon resonance analyses were performed comparing the affinities of 52^2 Cys variants with their parent antibodies. These analyses demonstrated that the haptenylated payloads bind their cognate derivatives in the same manner as parent antibodies.

**Thiol-containing payloads for hapten-positioned disulfide coupling**

Payloads for hapten-positioned disulfide coupling shall bind to the antibody binding pocket by the hapten, to be positioned for subsequent site-directed conjugation. Covalent coupling is achieved by disulfide linkage of the payload to 52^2 Cys in the rim region of the antibody. To achieve this functionality, payloads must fulfill the following criteria:

i. Payloads must contain hapten for positioning, thiol for disulfide coupling, and functional payload.

ii. The thiol must be connected to hapten without interfering with hapten binding, with linkers facing towards or from the rim region of the antibody. To achieve this functionality, payloads must be flexible and hydrophilic PEG3-residue (PEG3 = 12-amino-3,6,9-trioxododecanoic acid) (Dig-Cys-PEG-Cy5).

iii. The distance between cysteine and Cy5 has been elongated via a flexible and hydrophilic PEG3-residue (PEG3 = 12-amino-3,6,9-trioxododecanoic acid) (Dig-Cys-PEG-Cy5).

The distance between the C3 of Dig and Cα of Cys that can be spanned in Dig-Cys-Cy5 and in Dig-Cys-PEG-Cy5 by a maximally extended linker is ~21 Å. The distance between C3 of Dig to which the linker is attached and the thiol of 52^2 Cys at the rim region of the antibody is 11.5 Å. Thus, the linker is of sufficient length and due to its composition of sufficient flexibility to allow mutual access between the thiol of the payload and 52^2 Cys (Fig. 3E). The “distal” linker between the thiol and Cy5 is flexible enough to provide sufficient space between thiol and Cy5 to avoid interference with disulfide formation. Biot-Cys-Cy5 and Biot-Ser-Cy5 were generated following the same design principles (Fig. 4B), providing also sufficient space and flexibility for hapten binding and disulfide formation without payload interference. Control molecules contained a serine instead of the cysteine in the coupling position. In addition to fluorescent payloads, therapeutic peptide derivatives coupled (with and without thiol linkers) to hapten were generated following the same design principles (Fig. 4C).

**Covalent coupling of Hapten-Cys-Cy5 to 52^2 Cys containing IgG**

Complexes between hapten-Cys-Cy5 and anti-hapten 52^2 Cys IgG were generated by incubating antibodies with payload in a 2:5:1 molar ratio (slight payload excess as one IgG has 2 hapten binding sites, see Materials and Methods for details of the charging reaction). The products of this reaction were subsequently analyzed to evaluate if thiol-containing payloads become covalently attaching. The fluorescence of the payload Cy5 enables simple and robust detection of disulfide-mediated payload coupling by SDS-PAGE: samples are heated in SDS-containing sample buffer that denatures the hapten binding pocket. Thereafter, the samples are separated by nonreducing or reducing SDS-PAGE followed by detection of the payload via its fluorescence. These analyses (Fig. 5A for Dig and Fig. 5B for Biot) demonstrate that payloads become separated from the antibodies that do not contain 52^2 Cys during SDS-PAGE and appear predominantly as low
molecular weight signals at the bottom of the gels. This indicates that (as expected) denaturing sample preparation and SDS-PAGE disintegrates the hapten binding pocket and hence separates antibody and payload. Payload separation was observed not only for complexes of hapten-Cy5 with wild-type and 52\(^{+2}\) Cys containing IgG, but also when hapten-Cys-Cy5 was complexed with the cognate wild-type antibody. In contrast, hapten-Cys-Cy5 and hapten binding 52\(^{+2}\) Cys IgG form complexes that are additionally linked by a disulfide. In denaturing nonreducing SDS-PAGE, the fluorophores of such complexes are detectable as signals attached to the large IgG, indicating covalent linkage between payload and protein (Fig. 5). Separation of the Dig or Biot payloads from their respective protein partners occurs under reducing conditions. This confirms that payload and antibody are disulfide-bonded and become separated by reduction. Mass spectrometry analysis of Biot-Cys-Cy5 in complex with the antibody revealed that the payloads became covalently coupled to the antibody in more than 95% of the molecules (Table 2). We conclude from these experiments that the hapten-thiol payload becomes covalently linked via a disulfide to 52\(^{+2}\) of the hapten binding antibody.

Site-directed coupling involves hapten positioning and spontaneous redox shuffling

Effective formation of covalent complexes between hapten-thiol payloads and cognate 52\(^{+2}\) Cys IgG derivatives were observed for all hapten/antibody pairs that we tested (Fig. 5, Table 2). To test whether this disulfide connection is triggered by specific hapten-mediated positioning of the payload, additional coupling experiments were performed using “wrong” hapten-antibody pairs as controls: specific and effective covalent payload linkages with antibodies can only be achieved with matched hapten-antibody pairs (Dig with anti-Dig, Biot with anti-Biot; Fig. 6A). Combining Biot-Cys-Cy5 with anti-Dig 52\(^{+2}\) Cys does not trigger effective formation of covalent payload-antibody complexes (only some nonspecific background can be seen in the wrong combination, with most of the thiol payload unreacted), and complete coupling and lack of free payload is observed with the correct payload-antibody combination. Thus, hapten-mediated specific positioning of a thiol-containing payload is required for effective covalent disulfide linkage between both.

Figure 4. Composition of payloads for hapten-mediated site-directed disulfide coupling. A) Digoxigenylated fluorescent payloads are: Dig-Cy5, Dig-Cys-Cy5, Dig-Cys-PEG-Cy5; (B) Biotinylated fluorescent payloads are: Biot-Cys-Cy5, Biot-Ser-Cy5, (C) Hapten-coupled peptides are: Biot-Ser- and -Cys-containing derivatives of moPYY and Fluo-Cys-containing derivative of moPYY.
What drives the formation of disulfides between hapten-positioned payload and the 52\(^{22}\) Cys containing IgG? We generated hapten(Dig)-thiol-payload-antibody complexes in buffers with different amounts of oxidizing and/or reducing components. Previous observations indicated that coupling of payloads to antibodies via extra cysteines requires limited reduction (e.g., THIOMABS) because engineered cysteines added to antibodies that are produced in mammalian cells are not present with free sulfhydryl (SH) groups. Instead, they are disulfide-bonded to cysteines or glutathiones to which they were exposed while being expressed in mammalian cell culture. The 52\(^{22}\) Cys mutation of the IgG derivatives that we applied in these studies presents itself, because it is produced in a mammalian expression system, also not with a free SH side chain, but instead is disulfide-bonded to homocysteine as determined by MS analyses (see Materials and Methods). Figure 6B shows that, despite of the absence of free SH groups on the antibody, best coupling results are obtained under conditions that did not involve any redox system. Addition of redox buffers decreased the coupling efficacy and led also to undesired partial reduction of the antibodies at hinge disulfides and disulfides between HCs and LCs. This leads to partial dissociation into half-antibodies, HCs, and LCs (Fig. 6B). The specific disulfide bonding of the payload to the “blocked” cysteine occurs only when the hapten of the thiol-containing payload is bound to the 52\(^{22}\) Cys-containing antibody as described above. Hapten binding positions the thiol of the payload in spatial proximity to the blocked cysteine, enabling disulfide

### TABLE 2. Mass spectrometry analyses of antibody payload complexes reveal the content of covalently linked payload within the antibody-payload complexes

| Antibody | IgG thiol | IgG + 1 Cys/homocys | IgG + 2 Cys/homocys | HC + LC | Other\(^b\) |
|----------|-----------|---------------------|---------------------|--------|----------|
| <Dig> Cys | – | <6% | >94% | – | – |
| <Dig> Cys reduced | – | – | – | >90% | <10% |
| <Biot> Cys | – | – | >97% | – | <3% |
| <Biot> Cys reduced | – | – | – | >90% | <10% |
| <Fluo> Cys | – | – | >85% | – | <15% |
| <Fluo> Cys reduced | – | – | >90% | – | <10% |

\(^{a}\)Observed mass difference of 1 Cys/homocys was 128 (Dig), 254 (Biot), or 254 (Fluo). \(^{b}\)“Other” was observed as a mixture of adducts, Gly and Lys heterogeneity, LC cysteinylation, Tris(2-carboxyethyl)phosphine (TCEP), artifacts or glycation. The <15% other of <Fluo> Cys was predominantly adducts.
shuffling between antibody and payload. This results in effective attachment of the payload to the antibody at the predefined position without formation of aberrant coupled side products.

Site-directed covalent coupling improves complex stability in vivo

One objective to covalently link payloads to hapten binding antibodies is to increase the stability between payload and antibody in vivo. We have previously shown that noncovalent connections between antibodies and haptenylated payloads can be applied to prolong the serum half-life of small compounds and peptides (17). In these experiments, we observed a profound increase of the serum half-life of the payload in animals in comparison with noncovalently linked complexes.

Site-directed covalent coupling of therapeutic peptides

We have used for platform setup Cy5 as a “model payload” because its fluorescence allows a robust and rapid evaluation of coupling efficacy. To demonstrate that the platform enables covalent coupling of other payloads, we generated hapten-thiol-peptide derivatives whose design followed the same rules as described for Biot-thiol-Cy5. One of these payloads is Biot-Cys-moPYY (Fig. 4C), an analog of peptide tyrosine tyrosine or pancreatic peptide YY [PYY (3–36) analog] (17). This peptide binds to the Y2 receptor (28–30) and thereby modulates energy homeostasis by balancing food intake. PYY derivatives inhibit gastric motility and reduce appetite (28–30) and may thereby be useful for treating type 2 diabetes or obesity. However, PYY-derived peptides have a short serum half-life in vivo, and hence therapeutic applications would benefit from PK extension technologies.

Coupling of the Biot-Cys-moPYY peptide to the 52+2 Cys Biot-antibody under similar conditions as those used for
the coupling of Biot-Cys-Cy5 resulted in very effective site-specific covalent linkages between peptide and antibody. In the same manner as observed for the payload Cy5, the disulfide formation occurred with high efficacy by spontaneous shuffling, without the need for addition of redox reagents (see Materials and Methods). Mass spectrometry analyses demonstrated coupling efficiencies of close to 100% (Table 2) for covalent connection between 52\textsuperscript{Cys} and Biot-Cys-moPYY. The peptide Dig-Cys-moPYY is very similar in composition with Bio-Cys-moPYY but contains Dig instead of Bio as hapten. This peptide also undergoes spontaneous disulfide coupling to its cognate Cys52\textsuperscript{Cys}-containing antibody, but with somewhat reduced efficacy (80% bicoupled and 20% monocoupled; Table 2). An explanation may be that (at least for some payloads) hapten payload combinations can affect coupling efficiencies, possibly by modulating biophysical properties of the combined payload. Another haptenylated peptide is Flu-Cys-moPYY (Fig. 4C), which is similar in composition to Biot-Cys-moPYY, but which contains Fluo instead of Biot as hapten. This peptide can be coupled to its corresponding 52\textsuperscript{Cys}-mutated Fluo binding antibody (Fig. 2). Mass spectrometry analyses demonstrated again coupling efficiencies of close to 100% (Table 2) for covalent connection between <Fluo> Cys and Flu-Cys-moPYY as payload. We conclude from these experiments that the hapten-thiol payload platform works not only for small molecules (example Cy5) but also for larger molecules such as peptides.


diagram

**Figure 7.** Hapten-driven covalent coupling of Cy5 via a disulfide bridge increases the serum half-life of antibody-coupled payload. A) The serum half-life of noncovalently complexed Dig-Cy5 is significantly prolonged (solid rhombuses) compared with uncomplexed Dig-Cy5 (triangles), but shorter than the serum half-life of the Dig binding antibody (open rhombuses). B) The serum half-life of covalently complexed Dig-Cys-Cy5 (solid squares) is significantly prolonged compared to uncomplexed Dig-Cy5 (triangles), and the same as the serum half-life of the Dig binding antibody (open squares). The total serum levels of covalently linked Dig-Cys-Cy5 are larger than those of noncovalently complexed payload. C) The serum half-life of noncovalently complexed Biot-Cy5 is significantly prolonged (solid rhombuses) compared to uncomplexed Biot-Cy5 (crosses), but shorter than the serum half-life of the Biot binding antibody (open rhombuses). D) The serum half-life of covalently complexed Biot-Cys-Cy5 (solid squares) is significantly prolonged compared with uncomplexed Dig-Cy5 (crosses), and the same as the serum half-life of the Biot binding antibody (open squares). The total serum levels of covalently linked Biot-Cys-Cy5 are larger than those of noncovalently complexed payloads.

Separation of antibody and disulfide-connected payload in vesicular compartments

Hapten-positioned disulfide-connected payloads can be delivered to and into cells by bispecific cell-targeting antibodies (bsAbs) that carry a modified hapten binding entity as second specificity. Are payloads that are delivered by bsAb also released within cells by reduction of the disulfide that connects the payload? To address this question, we generated a bsAb that bind to the LeY antigen and simultaneously carries anti-Biot Cys52\textsuperscript{Cys} binding entities as disulfide-stabilized single-chain Fv additions (Fig. 8A).
Charging with Bio-Cys-Cy5 generates disulfide-connected bsAb-payload complexes in the same manner as monospecific anti-Biotin Cys52 (Fig. 8B). The LeY antigen is abundant on breast cancer cells and internalizes, and LeY binding antibody derivatives have previously been shown to deliver payloads to and into cells (such as MCF7) (24). Confocal microscopy analyses using Alexa-labeled secondary antibodies to detect bsAb and fluorescence to detect the Cy5 payload show that bsAb payload binds to and internalizes into MCF7 (Fig. 8C). However, bsAb and payload do not remain stably connected in cells as separation of payload from the antibody can be observed over time (e.g., already 6 hours after application). The separated bsAb is visualized in these assays by secondary antibodies that require bsAb stretches/domains to be intact to be detected. It is therefore unlikely that bsAb degradation is the major cause of the intracellular payload release. It is reasonable to assume that the observed intracellular payload release is predominantly triggered by intracellular reduction and hapten dissociation.

**DISCUSSION**

52^2 in the “rim region” is a general position for disulfide coupling of haptenylated payloads: the position needed to be in close proximity to the hapten-binding pocket, close to the linker between hapten and payload exiting the binding pocket, yet without interfering with hapten binding itself. The latter objective, avoiding positions that contribute to antigen binding, could be easily met by avoiding residues within classically defined CDRs (e.g., according to the Kabat numbering scheme) (23). However, avoiding CDRs counteracts the requirements proximity to the linker-thiol and hapten-positioning site. We therefore defined the binding region of hapten binders not by adherence to the classic CDR definition, but rather by defining binding pockets and “rim regions.” The rim region of a hapten binder surrounds the pocket yet does not directly contribute to antigen binding. 52^2, which we chose for cysteine replacement and disulfide coupling, is positioned quite similar for the hapten binders that we analyzed (Fig. 3). It is part of CDR H2, yet does not contribute to hapten binding. Analyses of 140 additional hapten binding structures from the PDB database confirmed that with one single exception, 52^2 locates in the rim region of all binders without itself being part of the binding pocket or interacting with haptens (Supplemental Data). Thus, 52^2 should be a truly universal position for disulfide coupling of haptenylated payloads, for many different haptens and corresponding hapten binding antibodies. In fact, based upon this principle that we initially derived from the structures of anti-Dig and anti-Fluo antibodies, we originally introduced the 52^2 Cys mutation into the Biot binding antibody in a blind manner several months before having access to the structure that we generated for this antibody.

The reaction that enables stable payload connection to 52^2 Cys-containing antibodies is hapten-positioned spontaneous disulfide shuffling: payloads with thiol-containing linkers were designed to place the thiol into proximity to 52^2, provided the hapten binds into its pocket. Such payloads can be chemically synthesized and purified to contain free thiols. In contrast, recombinant antibodies are produced in eukaryotic cells where they become secreted...
into culture supernatants. Because of the cellular production and secretion process, such antibodies even if they carry extra unpaired cysteines do not harbor free thiols. Instead, exposed thiols are present in form of disulfides (i.e., attached to other cysteine, homocysteine, or glutathione). Mass spectrometry analyses confirmed that our antibody preparations contained no accessible thiol. The fact that 52^{2+} Cys is blocked (and hence not reactive) is favorable for the production and handling of the antibody derivatives, as reactive surface cysteines would generate undesired covalent antibody dimers or multimers.

We also observe efficient covalent payload coupling to the blocked 52^{2+} Cys by a simple mix-and-incubate reaction in buffers that do not contain reducing or oxidizing or redox-shuffling reagents. This indicates that covalent attachment occurs via a spontaneous local redox shuffling reaction specifically between 52^{2+} Cys and the thiol-containing payload. Alkylation of the Cys, in fact, may be required for the coupling as new disulfide bonds normally arise when an unpaired Cys thiol attacks one of the Cys sulfurs of an existing disulfide bond. This “normal” reaction is mimicked by the free Cys of the hapten payload attacking and shuffling with the Cys-homoCys of the antibody. A scheme for the directed coupling reaction is shown in Fig. 9: binding of the hapten to the antibody positions the thiol into close proximity to the disulfide-blocked Cys. This triggers a redox-shuffling reaction between payload and 52^{2+} Cys, liberating a free cysteine from the disulfide. The reaction is directed toward payload-antibody linkage because (in contrast to the haptenylated payload) the free cysteine is not tethered to the antibody. Redox-shuffle back-reactions (Cys-SH reducing IgG-S-S payload) are possible but will not result in payload loss as the hapten still keeps it in place. A final purification step after the coupling reaction (e.g., SEC) can then be applied to remove the liberated cysteine and thus prevent any possible back-reaction and thereby “fix” the payload on the antibody.

Covalent linkage of hapten-positioned payloads is a general platform for site-directed attachment of payloads to antibodies and can be applied for targeted payload delivery. We have demonstrated the concept and the universality of the chosen position for 3 different hapten binders (and many more may be suitable, see Supplemental Data and Table). Payloads that we have attached include small molecules as well as peptides of intermediate size. The concept may also be applicable to large entities such as proteins, nucleic acids or nanoparticles, as long as they contain a free thiol to access 52^{2+}. For targeted payload delivery, hapten-binding entities that carry this mutation can be attached to cell or disease tissue-targeting antibodies. Such bispecific antibodies carry attached payloads to target sites and release payloads upon reduction (e.g., after internalization in reducing vesicular compartments as shown in Fig. 8). Thus, such bispecific antibody derivatives may be useful to deliver cytotoxic payloads to target cells such as tumors, to achieve antibody-drug conjugate-like functionalities.

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Figure 9. Hapten-positioned redox-shuffling at a blocked 52^{2+} Cys of that of the hapten binder connects the thiol-containing payload to the antibody. The reaction is driven toward payload coupling by the hapten occupancy of the antibody, which consistently keeps the payload thiol in close proximity to 52^{2+}, and the release of the liberated homoCys from the antibody (which limits back-reactions).
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