

Supplementary Methods

DARPin: Cloning, expression and purification

The DARPin H10-2-G3(27) (abbreviated here as G3) was cloned either fused with a N-terminal Met-Arg-Gly-Ser-(His)₆ tag alone or in combination with a C-terminal (myc)₅ tag or a C-terminal hemagglutinin tag into a derivative of pQE30 (QIAGEN).(32) In order to generate biotinylated monovalent DARPins the coding sequences were subcloned into pBD001, a derivative of pAT224 (GenBank accession number AY327139), as fusion with an N-terminal AviTag for enzymatic biotinylation and a C-terminal (His)₆ tag for purification, but devoid of the protein D fusion. The bivalent DARPin G3-linker-G3 and the trivalent DARPin G3-linker-G3-linker-G3 were cloned into the vector pQI_Bi_2_2 (Kawe et al., manuscript in preparation) fused to an N-terminal AviTag and a C-terminal (His)₆ tag for purification. In these constructs, the individual DARPins are spaced by a flexible (GGGS)₄ linker. After verification of the correct DNA sequence, DARPins were expressed in the *E. coli* strain XL1-Blue (Stratagene). For biotinylation of DARPins the *E. coli* biotin ligase encoded by the plasmid pBirA (Avidity) was co-expressed.(33) Cells were harvested 4-5 h after induction with 0.5 mM IPTG at 37°C in 2YT media containing 50 µM biotin. Cells were lysed using sonication and DARPins were purified from the soluble cytoplasmic fraction via a Ni-NTA column, followed by dialysis against phosphate-buffered saline (PBS). For the comparative study of DARPin properties, DARPins were additionally purified over a Superdex-200 16/60 gel filtration column (GE Healthcare). Pooled fractions were further purified over a monomeric avidin column (Pierce). Elution was performed with 5 mM biotin, and free biotin was removed using a NAP-5 desalting column (GE Healthcare) followed by extensive dialysis.

Affinities given in Supplementary Table 1 were determined for the monomeric DARPins containing an N-terminal Met-Arg-Gly-Ser-(His)₆ tag (G3(27), G3-D(27), G3-AVD(27), G3-HAVD(27); H6-3-B3(26); H14R, Jost and Plückthun unpublished data). The avidity of the multivalent constructs G3-linker-G3 and G3-linker-G3-linker-G3 depends on the surface density of HER2 and thus on the cell type, but on HER2-overexpressing cells G3-linker-G3 was found to show no measurable off-rate,(29) indicating strong bivalent binding on cells.

Additional information of DARPIn-Histochemistry of HER2

Additionally, we tested also further signal amplification using a secondary antibody against biotin (Jackson ImmunoResearch Labs, No. 200-002-096) in a dilution 1:1000 followed by incubation with secondary antibody coupled to a HRP-coupled polymer (Leica, Bond Polymer Refine Detection, No. DS9800). This resulted in a moderate amplification of the membranous staining, however leading to an unspecific cytoplasmic staining in some tumors. Alternative detection methods were also evaluated, including the detection of DARPIn G3 in the Met-Arg-Gly-Ser-(His)₆ tagged form with a anti-RGS(His)₄ mouse monoclonal antibody (QIAGEN GmbH, No. 34610) in a dilution of 1:100. However, even at concentration of 25 µg/ml, no signal was detected. The same DARPIn containing a (myc)₅ tag was detected with a mouse monoclonal anti-myc tag antibody (clone 9B11, Cell Signaling Technology, No. 2276) in a dilution of 1:1000. However, this resulted in nonspecific staining. Both anti-RGS(His)₄ and anti-myc tag antibodies were detected with a secondary anti-mouse antibody, coupled to a HRP-coupled polymer (Leica, Bond Polymer Refine Detection, No. DS9800). In addition, the DARPins G3 and H6-3-B3 fused with a hemagglutinin (HA) tag were detected with a mouse anti-HA antibody (Roche Diagnostics, clone 12CA5) in a 1:4000 dilution, and detection was carried out with a

biotinylated anti-mouse antibody and a streptavidin-HRP conjugate (LSAB2 kit, Dako).

Histological analysis of immunohistochemistry, DARPIn-Histochemistry and fluorescent *in situ* hybridization

The interpretation of the membranous staining obtained by immunohistochemistry and DARPIn-Histochemistry as well as the fluorescence signals by fluorescent *in situ* hybridization was carried out according to the recommendations of the American Society of Clinical Oncology and the College of American Pathologists.(1)

Histological staining patterns for immunohistochemistry and DARPIn-Histochemistry were analyzed as follows: a positive result of 3+ was defined as a uniform, intense membrane staining of > 30% of invasive tumor cells. Complete membrane staining that was circumferential in at least 10% of cells was scored as 2+. Weak, incomplete membrane staining of any proportion or complete membrane staining in less than 10% of cells was scored as 1+, and no staining in invasive tumor cells as 0. For antibody staining, a carcinoma was considered as HER2 overexpression-positive if a staining pattern of 3+ was present, intermediate (termed equivocal for overexpression) in the case of 2+ and negative in the case of 0-1+ according to the guidelines.(1) For the DHC, a positive HER2-status was defined as 2-3+, equivocal as 1+ and absent staining as 0 (see Results). The interpretation of the fluorescence signals obtained by FISH was carried out as follows: negative for HER2 amplification was defined as less than 4.0 *HER2* gene copies per nucleus or a fluorescent *in situ* hybridization ratio (*HER2* gene signals to chromosome 17 signals) of less than 1.8. A positive result was defined as more than 6 copies per nucleus or a FISH ratio of more than 2.2. The remaining intermediate cases were scored as equivocal.

Statistics

Kendall's tau-beta was used for correlating ordinal expression variables (0, 1-3+) with amplification status (negative, equivocal, positive) and Wilcoxon signed rank test for evaluating differences within these variables, as depicted in Table 1. In addition, for scored data (negative, equivocal, positive) in Table 2 a measure of agreement between methods was determined using kappa statistics.

In order to verify if DARPIn or antibody can reliably detect an equivocal and/or positive amplification status, receiver operating characteristic analysis was performed on the scored data sets. Specificities and sensitivities between DARPIn-Histochemistry and immunohistochemistry were compared using the McNemar-test. Results were considered statistically significant if p-values were 0.05 or less.

Supplementary Tables and Figures

Supplement to Fig. 1. A: The model of the HER2 dimer was assembled from the known structures of the extracellular domains I-IV (ECD), the transmembrane domain (TM) and the kinase domain dimer. The membrane-proximal domain IV, epitope of the DARPins investigated here is labeled. Linker sequences presumed to have no rigid structure are denoted by black lines, and the length is indicated in amino acids (aa). The extracellular (ex.) and cytoplasmic (cyt.) sides are labeled and the dashed line indicates the plasma membrane. No structural information is available for the C-terminal tail of the last 226 aa. **(B)** Bivalent IgG (150 kDa (kilo Dalton)), with the paratope overlaid in yellow. **(C)** DARPIn structure shown as a ribbon model (above) and a space-filling model (below). While the molecular weight of the DARPIn G3 used in this study is only 14.5 kDa, the size of its paratope

(overlaid in yellow) is comparable to that of an antibody. Each repeat of the DARPin consists of 33 amino acids forming a β -turn followed by two antiparallel α -helices. The internal repeats are randomized in the library and they can be evolved for new target specificity, while the repeats at the N- and C-terminus are important for maintaining the fold of the protein. The unstructured N-terminal AviTag for biotinylation (left) and the C-terminal His Tag for purification (right) are indicated. Other DARPins used as controls in this study (H6-3-B3 and H14R) have one more internal repeat and thus a MW of 18.5 kDa. **(D)** Bivalent DARPin G3-linker-G3 with a size of 31.2 kDa. The flexible linker of 20 aa allows the DARPin domains to adjust and permit independent binding to lead to a bivalent interaction. **(E)** Trivalent DARPin (G3-linker-G3-linker-G3 with a size of 45.8 kDa). The flexible linker is the same as in (D). The model of the HER2 dimer is based on the PDB file 1N8Z using the relative orientation of the EGF receptor dimer from the PDB file 1M0X. The model of the kinase domain is based on the PDB file 2G52 and the antibody model on the PDB file 1IGT. The DARPin model was based on the PDB file 2JAB. The linkers and tags are modeled in random conformation. Models are presented to scale.

Supplementary Fig. 1. Consecutive tissue sections of an HER2-non-amplified and – amplified carcinoma incubated with the HA-(26) or biotin-tagged G3 using either the automated staining system or the protocol published by Zahnd et al.(26) HA-based detection compared to biotin resulted in a slightly increased membranous signal in amplified cells, however, paralleled by the presence of strong unspecific cytoplasmic staining. Automated pretreatment of the tissue sections resulted in an increase in the

intensity of the specific, membranous signal when compared with the previously described protocol,(26) (all 200x magnification, bar represents 15 μ m).

Supplementary Fig. 2. Consecutive tissue sections of the same carcinomas as depicted in Supplementary Figure 1 incubated with the HA-(26) or biotin-tagged H6-3-B3 using the automated staining system and the identical conditions as for G3 detection (see Supplementary Fig.1). Using a concentration of 5 μ g/ml of the DARPin H6-3-B3 no signal was detectable for either detection method. Raising the DARPin concentration to 50 μ g/ml both detection methods gave a specific membranous HER2 staining pattern as previously described for the HA detection method,(26) (all 200x magnification, bar represents 15 μ m).

Supplementary Table 1. Characteristics of biotinylated DARPin clones against HER2 used in this study.

DARPin clone	MW (kDa)	Epitope	Avidity	Affinity
G3*	16.4	ECD 4	monovalent	90 pM
G3-D*	16.4	ECD 4	monovalent	1.48 nM
G3-AVD*	16.4	ECD 4	monovalent	10.2 nM
G3-HAVD*	16.4	ECD 4	monovalent	269 nM
H14R ^o	19.95	ECD 4	monovalent	220 pM
H6-3-B3 [#]	20.1	ECD 4	monovalent	28 nM
G3-li-G3	31.2	ECD 4	bivalent	\$, §
G3-li-G3-li-G3	45.8	ECD 4	trivalent	nd

*reference 26; ^oJost and Plückthun, unpublished data; [#]reference 25; ^{\$} reference 30, [§]no measurable off-rate

Affinities were measured with DARPin variants containing a tag different from that presented in this study, but in which all residues of the DARPin sequence are identical.

nd: not determined.