TOOLBOX: Strep-Tagged nano-assemblies of antibody-drug-conjugates (ADC) for modular and conditional cancer drugging

ELISA TREMANTE[1], LEONARDO SIBILIO[2,7], FABIO CENTOLA[2,8], NADINE KNUTTI[3,9], GERD HOLZAPFEL[4], ISABELLA MANNI[5], MATTEO ALLEGRETTI[1], PAOLO LOMBARDI[6], GIUSEPPE SALVO[2,10], LOREDANA CECCHETELLI[2], KARLHEINZ FRIEDRICH[3], JOACHIM BERTRAM[4] and PATRIZIO GIACOMINI[1]

[1]Oncogenomics and Epigenetics, IRCCS Regina Elena National Cancer Institute, I-00144 Rome; [2]Italian Biochemical Institute (Ibi) G. Lorenzini, I-04011 Aprilia, Italy; [3]Institute of Biochemistry II, University Hospital Jena, D-07740 Jena; [4]IBA GmbH, D-37079 Göttingen, Germany; [5]SAFU, IRCCS Regina Elena National Cancer Institute, I-00144 Rome; [6]NaxosPharma, Novate Milanese, I-20026 Milan, Italy

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Abstract. Herein, we describe TOOLBOX, a 3-step modular nano-assembly targeting system that permits the combinatorial exchange of antibody specificities and toxic payloads, introducing modularity in antibody-drug conjugate (ADC) manufacturing. TOOLBOX integrates 3 building blocks: i) a recombinant antibody fragment (that in the selected setting targets the proto-oncogene ERBB2) genetically fused to an 8 amino acid Strep-Tag®; ii) a multivalent protein adapter, called Strep-Tactin®; iii) two anticancer agents, e.g. DNA nanobinders and the maytansinoid DM1, both carrying a chemically attached Strep-Tag that reversibly turns them into inactive prodrugs. Stoichiometrically optimized complexes of Strep-Tagged antibody fragments and drugs, bridged by Strep-Tactin, were specifically uptaken by breast cancer cells expressing ERBB2, and this unexpectedly resulted in conditional prodrug reactivation. A promoter-reporter system showed that TOOLBOX inhibited downstream ERBB2 signaling not only in ERBB2-overexpressing/-amplified SK-BR-3 cells grown *in vitro*, but also in ERBB2-low/non-amplified BRC230 triple-negative breast carcinoma cells xenotransplanted in nude mice. Thus, TOOLBOX is a modular ADC-like nano-assembly platform for precision oncology.

Correspondence to: Dr Patrizio Giacomini, Oncogenomics and Epigenetics, IRCCS Regina Elena National Cancer Institute, Via Elio Chianesi 53, I-00144 Rome, Italy
E-mail: patrizio.giacomini@ifo.gov.it

Present addresses: [7]Menarini Biotech, Via Tito Speri 12, I-00040 Rome, Italy; [8]Global Analytical Pharmaceutical Science and Innovation, Merck Serono S.p.A.; an affiliate of Merck KGaA, Darmstadt, Germany, Via Luigi Einaudi 11, I-00012 Guidonia Montecelio, Rome, Italy; [9]Institute of Clinical Chemistry and Laboratory Diagnostics, University Hospital Jena, D-07740 Jena, Germany; [10]External Quality Assurance (ExM), MSD Italia S.r.l., Via Vitorchiano 151, I- 00189 Rome, Italy

Abbreviations: ADCs, antibody-drug conjugates; T-DM1, trastuzumab emtansine; Ig, immunoglobulin; TNBC, triple-negative breast cancer; ATCC, American Type Culture Collection; HLA, human leukocyte antigens; mAb, monoclonal antibody; ScFv, single chain fragment of variable antibody regions; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PE, phycoerythrin; EMCS, N-(ε-maleimidocaproyloxy)succinimide ester; GFP, green fluorescent protein; DMF, dimethylformamide; CDR, complementarity determining regions; RLU, relative luminesce units

Key words: antibody-drug conjugates, precision oncology, ERBB2, HER2, self-assembling protein nanostructures, conditional drugging

Introduction

Antibody-drug conjugates (ADCs) combine the binding specificity of a monoclonal antibody with the toxic effects of a potent payload (1). The prototypal ADC trastuzumab-emtansine (T-DM1) is now the standard of care in advanced and adjuvant settings for breast cancer of the aggressive ERBB2 subtype (2,3). T-DM1 overcomes pharmacological resistance to trastuzumab (its unconjugated counterpart) and/or pertuzumab (another ERBB2-specific antibody) and/or small molecules (2). Moreover, it achieves better efficacy at lower dosages than naked antibodies, and permits reduction in the intensity of associated chemotherapy, drastically improving toxicity profiles altogether (1).

However, like any other ADC and anticancer drug, T-DM1 almost invariably induces pharmacological resistance by adaptive selection through: (a) tumor antigen/epitope loss, and (b) payload refractoriness (4). In principle, (a) and (b) could be effectively tackled by preparing as many ADCs as the number of possible combinations and permutations of all the actionable cancer antigens and known active drugs for a given tumor type. Then, these ADCs could be used in combination and/or in sequence. However, this combinatorial approach is neither conceivable nor applicable in practice, and for many reasons.

Each ADC is a unique product resulting from a dedicated and industrially demanding manufacturing process, whereby a specific immunoglobulin (Ig) is covalently attached (through a carefully designed chemical linker) to a selected cytotoxic agent (e.g. a microtubule inhibitor such as a maytansinoid) in defined stoichiometries (1). Therefore, an exponential expansion of the ADC arsenal would result in hyperbolic costs, issues in clinical trial design and regulatory clearance, and a likely biomanufacturing crunch.

To tackle some of these challenges, we took advantage of the degree of freedom that is only possible when designing objects on the nanoscale, and combined several useful drugging tools into a modular 'box' that, accordingly, was named 'TOOLBOX'. TOOLBOX (Fig. 1) is a multi-step platform comprising an affinity reagent of choice, that in the selected example is a single chain fragment of variable antibody region (ScFv) to the proto-oncogene product ERBB2, and a small anticancer drug that *per se* has no specificity for cancer cells. Both the ScFv and the drug are modified through the addition (by genetic engineering and chemical synthesis, respectively) of a so-called Strep-Tag, e.g. an 8-amino acid WSHPQFEK moiety. The two Strep-Tagged reagents may then be bridged by a universal multimeric adaptor protein (Strep-Tactin) that mediates the selective deposition of a cytotoxic nano-complex onto the surface of cancer cells. Since the affinity reagent and the drug are non-covalently linked, the two can be variably combined to generate a variety of tumor drugging systems.

This report describes the step-wise optimization of one such TOOLBOX drugging systems, and its use to redirect two different drugs onto ERBB2-high (susceptible to ERBB2 blockade) and ERBB2-low (putatively insensitive/resistant) human breast cancer cells, the latter grown as mouse tumor xenografts.

Materials and methods

For details and full descriptions of the Materials and methods please refer to Data S1.

Cell lines. SK-BR-3 is a certified cell line obtained from the American Type Culture Collection (ATCC). BRC230 cells (5) belong to a triple-negative breast cancer (TNBC) subtype expressing low ERBB2 levels (6,7), and were obtained from the originators. Cell lines were identity verified by human leukocyte antigen (HLA) typing, as described (8), and were routinely assessed for mycoplasma.

ScFv W6/800, antibodies and reagents. The murine monoclonal antibody (mAb) W6/800 to ERBB2 and the corresponding ScFv are described (9,10). Trastuzumab and oertuzumab (Herceptin® and Perjeta® were generous gifts of Roche-Genentech. mAb 108 to the epidermal growth factor receptor (EGFR) was obtained from the ATCC hybridoma HB-9764. Recombinant human epidermal growth factor (EGF) was obtained from ImmunoTools GmbH. Human NRG1-β1/HRG1-β1 EGF domain was obtained from R&D Systems, Inc. PD168393 (Calbiochem/Merck) is an irreversible EGFR inhibitor.

Recombinant DNAs, Strep-Tag technology and ScFv expression. All the tagging platforms and reagents are commercially available from IBA Lifesciences, except those that were developed for the purpose for this study, as noted.

Standard TOOLBOX flow cytometry protocol. The standard 3-step TOOLBOX protocol involves successive incubations with: i) tagged ScFv; ii) either phycoerythrin-conjugated Strep-Tactin (Strept-Tactin-PE) or its Strept-Tactin-Mult-PE variant; and iii) Strep-Tagged green fluorescent protein (One-Strep-GFP). In the two-step protocol, the concentrations of the reagents were identical, but Strep-Tactin-PE and One-Strep-GFP were admixed. Two-color flow cytometry was carried out in a FACScan (BD Biosciences). For further details see Data S1.

Strep-Tagged drugs. The organic synthesis of DNA nanobinders (NAX compounds) and their tagged derivatives (NAXT) is described (11). For tagging, maleimide derivatives of NAX compounds were mixed in equimolar amounts with the WSHPQFEK peptide in the presence of dimethylformamide (DMF) at room temperature for 4 h. DMF was vacuum-evaporated and the tagged compound (typically about 50% of the total mix) was purified by HPLC. Molecular weight was confirmed by MALDI-MS. Emtansine (DM1; Abcam) was Strep-Tagged (DM1T) by adding an N-(ε-maleimidocaproyloxy)succinimide ester (EMCS; MW 308.29) linker (Thermo Fisher Scientific, Inc.) that introduces a 9.4 Å spacer arm. Design and synthesis of NAXT and DM1T were carried out by Dr Anette Jacob at Peps 4 Life Sciences (Peps4LS), Heidelberg, Germany.

TOOLBOX treatments in vitro. The antiproliferative activity of NAX compounds was tested by incubating SK-BR-3 cells for 45 min at 4˚C in 96-well plates with the indicated concentrations of the TOOLBOX components in 100 μl of complete medium. The plates were then moved to a CO_2 incubator and grown for 72 h. ^{3}[H]-Thymidine incorporation was measured (triplicates) during the last 4 h of growth. The activity of DM1T was assessed by assessing light emission of an ERBB2 pathway-dependent c-fos promoter (12)/NanoLuc® (Promega Corp.) luciferase reporter assay. The construct (details in Data S1) was stably transfected into SK-BR-3 and BRC230 breast carcinoma cells. Cells were pre-treated with the TOOLBOX components for 45 min at 4˚C, then grown for 72 h, lysed in the presence of the furimazine substrate, and assessed for bio-luminescence in a luminometer (13).

Animal experiments. nu/CD1 mice bearing BRC230-cfos xenotransplants were treated by tail vein (i.v.) injection of either ScFv or ScFV-mut4 (10 mg/kg), followed 1 h later by a pre-mix of Strep-Tactin-Mult (7.5 mg) and DM1T (2 mg/kg), as approved (prot. 665/2017-PR) by the Animal Welfare Section of the Italian Ministry of Health. This study was compliant with the 2010/63/EU directive. At selected time points, the substrate furimazine was i.v. injected under mild anaesthesia, as previously described (14), and light emitted by the NanoLuc reporter was imaged in a IVIS Lumina (Perkin Elmer). Additional details on anaesthesia and sacrifice may be found in Data S1.

Statistical analysis. GraphPAD Prism v.8.3 (GraphPad Software, Inc.) was used for statistical elaboration.

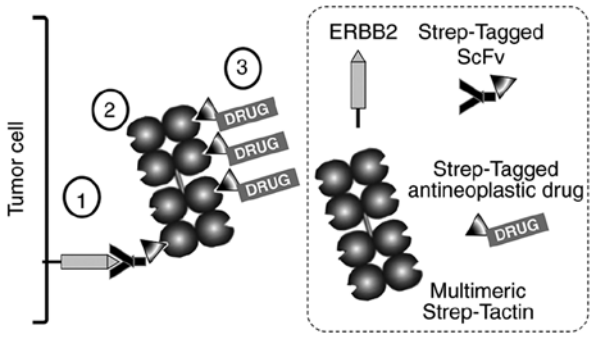

Figure 1. The TOOLBOX approach. ADCs deliver a toxic payload to cancer cells and have to be manufactured one by one. In contrast, TOOLBOX can mix-and-match the desired antibody specificity with a selected drug without covalent drug:antibody attachment. In the selected example, a tagged antibody fragment to HER2 (ScFv; step 1), a tag-specific multivalent protein adapter (Strep-Tactin, step 2), and a chemically tagged payload (step 3) bind sequentially, providing a flexible and modular tumor targeting strategy. ADCs, antibody-drug conjugates.

Results

Prior to attempting full nano-assembly, the TOOLBOX building blocks and protocols were optimized.

TOOLBOX: Optimizing step 1 (tagged ScFv). ScFv W6/800 to ERBB2 was selected for TOOLBOX since its antigen binding site is remarkably resilient to reduction and denaturation (10). Following removal of the leftovers of previous DNA manipulations, a clean 284 amino acid ScFv was obtained exclusively comprising murine Variable Heavy and Variable Light chain Ig sequences connected by a glycine-serine $(G_4S)_3$ linker. Following *in silico* codon optimization for mammalian expression, this DNA was whole-gene synthesized, cloned into the pESG142 and pESG144 double-tagging vectors (IBA Lifesciences), and expressed in a secretable form in CHO transfectants. The two resulting variants (ScFv142 and ScFv144) carry a Strep-Tag and a 6X His-tag arranged in the two possible orientations at the N- and C-termini of an otherwise identical ScFv backbone (Fig. 2A, left). ScFvs carrying mutated complementarity determining regions (CDR) (Fig. 2A, right) were also cloned in the pESG vectors.

Wild-type (WT) ScFv142 and ScFv144 were found to bind to ERBB2-overexpressing SK-BR-3 breast carcinoma cells similarly (Fig. 2B and C, magenta), but more weakly than the parental whole mAb W6/800 (green). This was expected, since previous Scatchard plot experiments demonstrated that monovalent binding causes a 4.5-fold drop in the ScFv equilibrium binding constant as compared to the whole murine antibody (10). Moreover, Fc epitopes (recognized by the FITC-labelled secondary antibody) are available on whole Igs but not the ScFvs. However, and remarkably, low ScFv binding could be compensated using either anti-tag reagents such as an FITC-labelled anti His-tag antibody (D and E), or a Phycoerythrin (PE) conjugate of a Streptavidin derivative named Strep-Tactin-PE (F and G). Both secondary reagents were able to drive onto breast cancer cells a substantial amount of fluorescence. Interestingly, although tags were accessible at both ends of ScFv142 and ScFv144, binding was stronger and less affected by ScFv dilution when the His-tag was mounted at the C terminus (compare E to D), and the Strep-Tag was mounted at the N-terminus (compare G to F), as in ScFv144. ScFv 144 performed better than ScFv142 (F/G vs. D/E) and was therefore selected for further studies.

Five ScFv144 variants (WT and 4 CDR mutants) were generated and tested in parallel by flow cytometry on ERBB2-high SK-BR-3 and ERBB2-negative A431 cells, using Strep-Tactin-PE as the secondary reagent (Fig. 2H-Q). As expected, A431 cells were unreactive (H-L), whereas parental ScFv144 displayed the strongest SK-BR-3 binding (M). Binding was gradually lost upon introduction of progressively more disabling CDR mutations (N, O and P, and see graphical ScFv representation on top of each panel and CDRs in panel A). However, consistent with a resilient antigen binding site, both CDR scrambling (exchange in amino acid order) and non-conservative substitutions at many positions were necessary for complete inactivation (ScFv144-mut4; Q; also see Table SI). In summary, Strep-tagged ScFv144 (hitherto ScFv) was identified as the affinity reagent of choice, and ScFv144-mut4 was set aside as a stringent negative control.

TOOLBOX: Optimizing steps 3 and 4. Two-color (red/green) flow cytometry was used to individually detect cell surface binding of the TOOLBOX components, and optimize their stoichiometry. As shown by the diagram in the lower left of Fig. 3, Strep-Tactin-PE was used to monitor TOOLBOX step 2 (adapter engagement) and, indirectly, step 1 (ScFv binding). A Strep-Tagged variant of the green fluorescent protein (GFP) called One-Strep-GFP was instead used to monitor step 3, e.g. it was used as a convenient technical surrogate of Strep-Tagged drugs. Two stepwise incubation formats were tested, namely: a) ScFv + an optimal Strep-Tactin:One-Strep-GFP pre-mix (2-step protocol); b) successive addition of ScFv + Strep-Tac tin + One-Strep-GFP (3-step protocol). These protocols are described in detail in Data S1.

As shown by a representative 2-step experiment, we consistently detected ScFv:Strep-Tactin-PE binding in the red channel (Fig. 3C compared to A and B). However, and paradoxically, One-Strep-GFP not only failed to bind, but it did abrogate red fluorescence (Fig. 3D compared to C, and see A and B), suggesting blocking of free Strep-Tactin valences. In the attempt to identify a critical stoichiometric window compatible with double Strep-Tactin engagement (with ScFv on the one hand and One-Strep-GFP on the other), first we increased Strep-Tactin-PE concentrations up to 8-fold, with no success (Fig. S1). Then, we diluted One-Strep-GFP, but also in this case no green signal could be rescued in either the 2-step protocol (Fig. S2A-D) or the 3-step protocol (Fig. S2E-H). However, and interestingly, a gradual rescue could be seen exclusively in the red (Strep-Tactin-PE) channel, and was more evident in the 3-step than the 2-step protocol (compare Fig. S2E-H to A-D), e.g. when One-Strep-GFP was added after washing away unbound Strep-Tactin-PE. This strongly indicated competition for a limiting number of binding sites of the adapter, e.g. an intrinsic shortage in Strep-Tactin-PE valence preventing the simultaneous engagement of ScFv and One-Strep-GFP.

Since Strep-Tactin is routinely polymerized at a predicted multiplicity of 4 (i.e. with 4 subunits carrying 4 nominal Strep-Tag binding sites), a novel Strep-Tactin was oligomerized

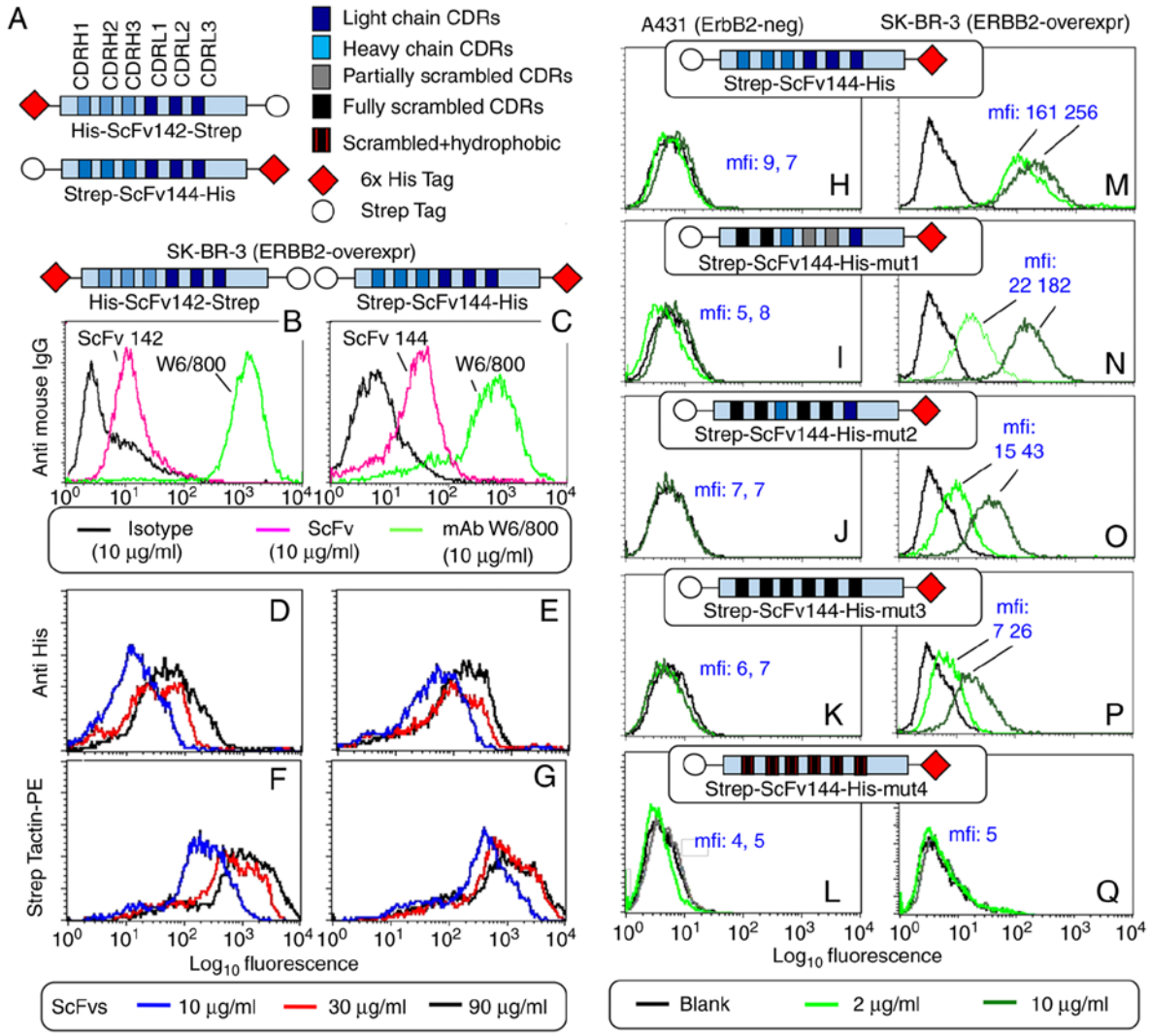

Figure 2. ScFvs to ERBB2: Variants and mutants. (A) Schematic outline of ScFv building blocks, including the murine Ig backbone, tags, and CDRs (both parental and mutated). Scrambled: Altered order of amino acid in each CDR. Scrambled + hydrophobic: Polar residues were turned into hydrophobic after scrambling. Additional details may be found in Table SI. (B and C) Schematic representation of the two parental, double-tagged ScFv constructs (ScFv142 and ScFv144), and flow cytometry testing on ERBB2-overexpressing SK-BR-3 cells. ScFv and parental antibody binding was revealed by FITC-labelled anti-murine and anti-human Ig secondary antibodies. (D-G) ScFv testing as above, but with anti-tag reagents at the indicated dilutions. (H-Q) Flow cytometry testing of ERBB2-negative and ERBB2-positive A431 and SK-BR-3 cells with ScFv144 and its CDR mutants (each graphically displayed on top of the relevant panels). CDRs, complementarity determining regions.

at a predicted multiplicity of 10 (Strep-Tact-Mult hitherto). Strep-Tact-Mult displayed a slightly reduced ability to detect ScFv binding (Fig. S3, compare B and C), but as expected it enabled double staining, particularly in the two-step protocol (Fig. 3H compared to E, F and G; and also compared with D). Binding was specific because addition of a non-fluorescent Strep-Tagged molecule (recombinant Streptamers, see Materials and methods) in 10x excess totally abrogated fluorescence in either or both the green and red channels (Fig. 3, compare J to C and K to I). Finally, unconjugated Strep-Tactin-Mult, like Strep-Tactin-Mult-PE, was found to also sustain multi-step TOOLBOX nano-complex formation (Fig. S4, compare D to C). This conclusively demonstrated specific interactions among the TOOLBOX building blocks, and a minimum nominal Strep-Tactin valence enabling TOOLBOX multimers. Then, we were ready to replace the optimized amounts of One-Strep-GFP with equimolar amounts of tagged drugs.

TOOLBOX assay development: Designing and optimizing Strep-Tagged anticancer drugs. Toxic payload delivery onto breast cancer cells was tested using the preferred 2-step protocol. Initially, DNA nanobinders (berberine derivatives) were selected as the preferred toxic payload since: a) they find application in many different neoplasms including breast cancer (11); and b) in-house expertise was available to design these chemical structures. Covalent modification with a bulky Strep-Tag (WSHPQFEK) was expected to irreversibly inactivate small drugs, including berberines. Then, we set out for a systematic synthesis and screening study aimed at selecting the rare berberine variants expected to retain their activity following Strep-Tagging.

Ten pairs of NAX compounds and Strep-Tagged NAXT counterparts were produced starting from the available (11) parent compounds. The organic synthesis of 4 of these compounds (NAX063, NAX073, NAX098, and NAX110) is outlined in Fig. S5. Their Strep-Tagged counterparts

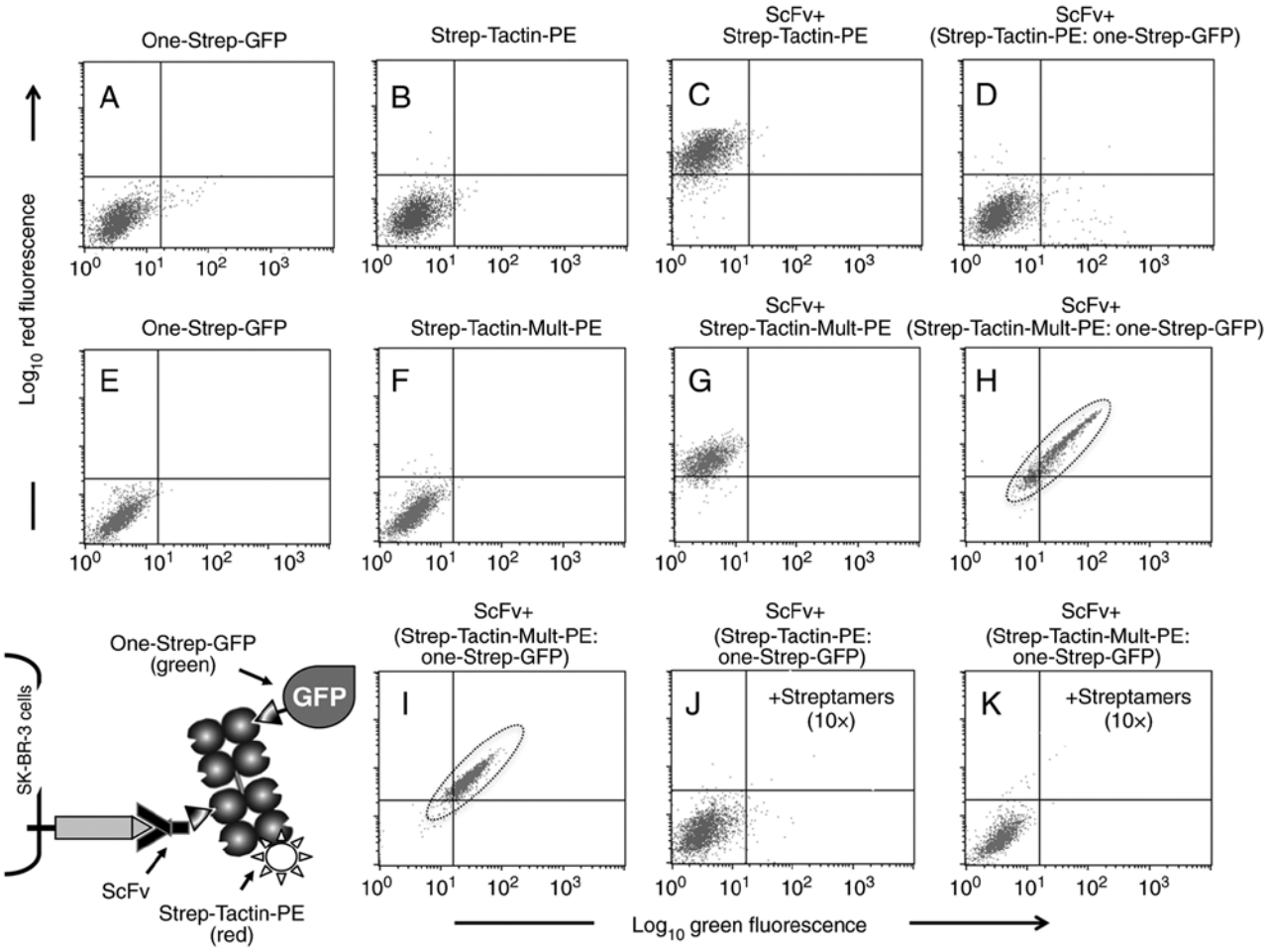

Figure 3. Production of Strep-Tactin multimers supporting the TOOLBOX protocol. SK-BR-3 cells were incubated with the standard amounts (see Data S1) of the TOOLBOX ingredients to obtain the formation of nano-assemblies containing ScFv, Strep-Tactin-PE and One-Strep-GFP, as depicted in the lower left. Strep-Tactin-PE and One-Strep-GFP were assessed by 2-color flow cytometry of SK-BR-3 cells in the red and green channels, respectively. Two distinct Strep-Tactin preparations were used: Strep-Tactin-PE and Strep-Tactin-Mult-PE, polymerized at average multiplicities of 4 (A-D and J) and 10 (E-I and K), respectively. Single-ingredient 2d plots (only one fluorescent reagent added) are shown as negative and positive controls in A-C and E-F. Panels D, G, H and I contain up to 3 distinct ingredients, added stepwise. When pre-formed (two ingredients) complexes are provided in a single incubation step, the name of the compounds is separated by a colon. Inhibition experiments in presence of excess tagged Streptamers are shown in J and K. Each of these experiments was performed at least five times with similar results. All the incubations were carried out on ice.

(NAX063T, NAX073T, NAX098T, and NAX110T) are displayed in Fig. S6. SK-BR-3 cells were treated for 72 h with two different concentrations of the NAX/NAXT pairs at micromolar concentrations, in line with the reported effective doses of these drugs (11), and then assessed for ^{3}[H]-Thymidine incorporation. Fig. 4A displays the selected NAX098/NAX098T pair, whereas results with all the 4 NAX compounds are shown and described in Fig. S7. As shown in Fig. 4B, NAX098 displayed the anti-proliferative activity typical (11) of this class of nanobinders (compare striped to white bars), but Strep-Tag addition resulted in complete or nearly complete inactivation (compare black and white bars). However, and unexpectedly, anti-proliferative activity was surprisingly reinstated when NAX098T was administered by the TOOLBOX protocol (stippled).

Thus, despite tagging turns berberins into inactive (pro) drugs, the process is reversible, e.g. NAXT compounds may be conditionally reactivated when they are incorporated into Strep-Tactin nano-complexes, possibly because TOOLBOX funnels them into an unknown cellular reactivation process. This makes the original TOOLBOX concept (Fig. 4C) feasible and (unexpectedly) even more appealing than anticipated.

TOOLBOX refinement: The ERBB2-cfos reporter, ERBB2-low cells and tagged emtansine. Next, to specifically assess the effects of TOOLBOX treatment on ERBB2 signaling, we developed an ERBB2-dependent genetic reporter system. In this construct, an improved luciferase (NanoLuc®) was cloned under the control of the c-fos promoter, a well-established downstream ERBB2 target (15). ERBB2 signaling was estimated by measuring the light emitted following conversion of the substrate furimazine. The construct was stably transfected not only in breast cancer cells of the ERBB2-subtype (SK-BR-3-cfos), but also in TNBC cells (BRC230-cfos) that do not host amplified ERBB2 (5). Low ERBB2 is a recognized feature of a fraction of TNBCs and is held responsible, among other ill-defined factors, for poor responsiveness of this molecular subtype to therapeutic anti-ERBB2 antibodies (16). The two cell lines were found to differ >10-fold in ERBB2 expression, as expected, but expressed similar EGFR levels

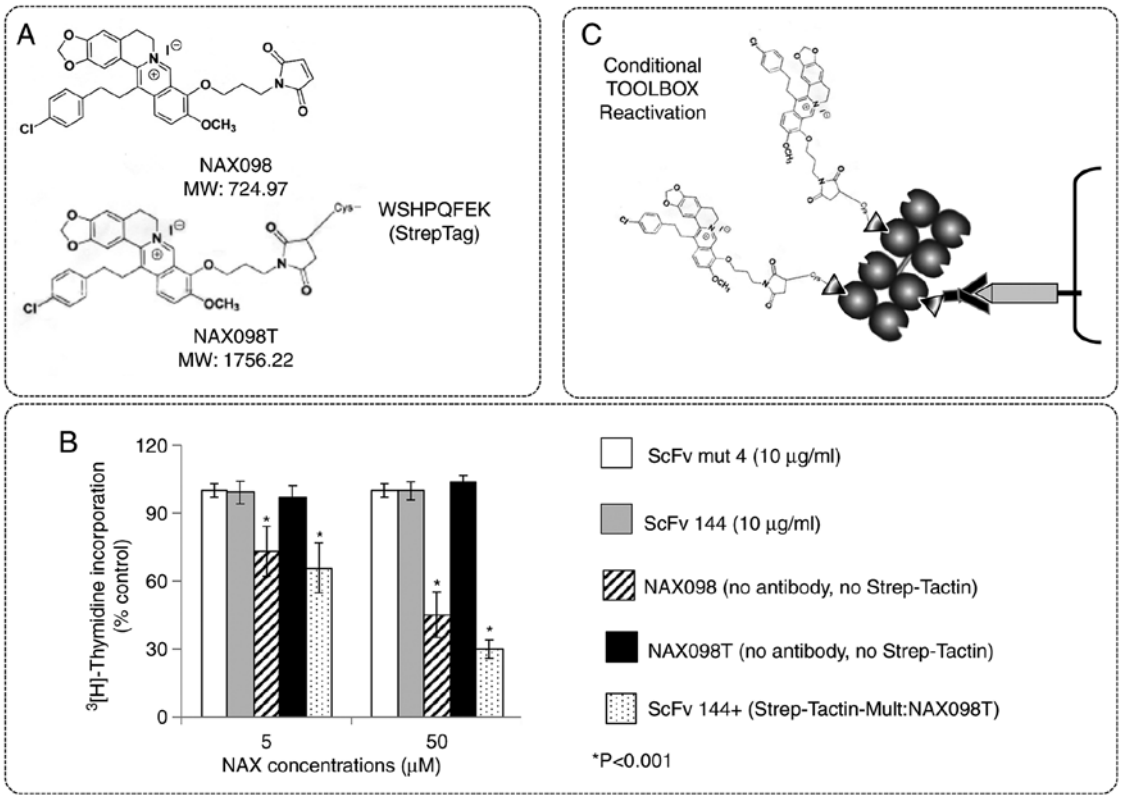

Figure 4. Antiproliferative effect of a Strep-Tagged DNA nanobinder. (A) Chemical structure of NAX098 and its Strep-Tagged derivative NAX098T. (B) ^{3}[H]-Thymidine incorporation was assessed during a 4-h radioactive pulse at the end of a 72-h 2-step treatment of SK-BR-3 cells with the TOOLBOX cocktail. Cells were pre-incubated at 4˚C in the presence of either the individual TOOLBOX components (white, grey, striped and black bars) or the full TOOLBOX cocktail (stippled), as described in Materials and methods. Results are expressed as percent of radionuclide incorporation as compared to untreated (complete medium) cells. Significance at Student t-test is noted. (C) Diagram of the experiment, that was performed three times with similar results. *p<0.001 compared to ScFv mut 4.

(Fig. S8). Accordingly, ERBB2-low BRC230-cfos transfectants exhibited more limited (but still detectable) response to the EGF, that mainly engages EGFR:ERBB2 dimers, and signaling was counteracted by treatment with the irreversible EGFR chemical inhibitor PD168393 (Fig. S9, compare A to B).

Having available an ERBB2-dependent promoter reporter system in an ERBB2-low cell line, we were particularly interested in assessing TOOLBOX performance under such demanding conditions. Then, we switched to a more potent Strep-Tagged drug. Emtansine (DM1) was selected, since this potent microtubule inhibitor is a preferred ADC payload, as in the prototypic ADC trastuzumab-emtansine (T-DM1). DM1 was attached to the Strep-Tag through a spacer (DM1T; Fig. 5A) and the resulting DM1T derivative was used to treat BRC230-cfos transfectants in a dose-response experiment (Fig. 5B). Cell viability was assessed by propidium iodide (PI) exclusion. With this method, the half-maximal inhibitory concentration (IC_{50}) of DM1 was in the sub-nanomolar range, as expected, and DM1T displayed limited residual activity. Because essentially inactive, DM1T could be incorporated in a 2-step TOOLBOX protocol at a concentration (2.5 nM) 5 times above the IC_{50} of DM1. Despite limited ERBB2 signaling, TOOLBOX treatment of BRC-230-cfos transfectants (Fig. 5C) counteracted not only NRG-1-mediated stimulation, but reduced light emission well below baseline (striped bar). Remarkably, DM1T was active (in the TOOLBOX protocol) at a concentration 10^3 times lower than NAX098T (compare with Fig. 4), and yet the pattern of conditional prodrug reactivation was very similar. We conclude that also a preferred ADC payload can be modified to fit the TOOLBOX approach.

TOOLBOX in vivo. Finally, a TOOLBOX experiment with DM1T was carried out *in vivo* on 6 nude mice (3 in the experimental group and 3 controls) bearing subcutaneous BRC230-cfos tumor xenografts. Treatment was carried out by a single tail vein injection of the TOOLBOX cocktail. The experimental group received ScFv + Strep-Tactin-Mult followed, 60 min later, by DM1T (see Materials and methods and Data S1 for details). Control littermates were treated as above but wild-type ScFv was replaced by equal amounts of inactive ScFv-mut4. ERBB2 activation was assessed by measuring light emission after *in vivo* furimazine injection at time 0 (before treatment) and 7 days later. In a single mouse, two additional intermediate determinations (24 and 48 h) were carried out. The effect on promoter activity was detected (2-fold to 8-fold reduction) in all mice (data not shown). In the mouse with multiple time points it was gradual and became fully evident on day 7 (Fig. 6). ERBB2 signaling either remained stable or slightly increased in the control mice (data not shown). Remarkably, TOOLBOX-treated and control-treated mice did not show appreciable signs of general toxicity upon injection of DM1T. Body weight remained fairly stable, e.g. from 26.1 (±0.4 SD) g at day 0, to 26.3 (±0.5 g) at day 7 in the active ScFv group, and from 26.7 (±1.2 g) at time 0 to 27.3 (±0. 9 g) at day 7 in the inactive ScFv-mut4 group.

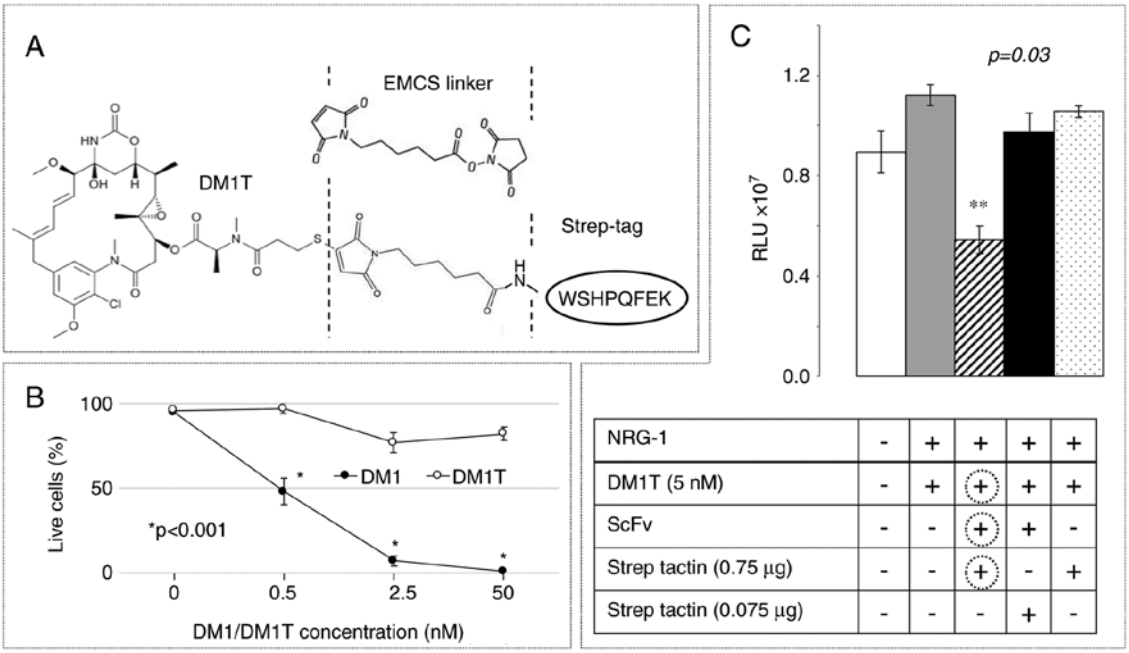

Figure 5. Antiproliferative effect of Strep-Tagged emtansine. (A) Chemical structure of emtansine (DM1), the EMCS linker, the Strep-Tag, and the tagged DM1T compound after covalent linkage of the three. (B) Dose-response of BRC230 cells treated at the indicated concentrations with DM1 and DM1T. Cell viability (%) was assessed in a flow cytometer by propidium iodide (PI) exclusion and is expressed as mean of viable cells ± standard Deviation of triplicates. Significance was calculated by the 2-tailed Student t-test. (C) ERBB2-low BRC230-cfos transfectants were pre-incubated at 4˚C with the indicated components of the TOOLBOX cocktail and then grown for 72 h in the presence and absence of the RTK ligand NRG-1. Relative luminesce units (RLU) emitted by the c-fos reporter was assessed in a luminometer. Controls are obtained by omitting (or reducing the concentration of) single crucial reagents. The components of the full TOOLBOX recipe at the optimal dosage are circled. Significance at Student t-test is noted in B and C. These experiments were performed three times with similar results. *p<0.01 compared to DM1T; **p=0.03 compared to NRG-1.

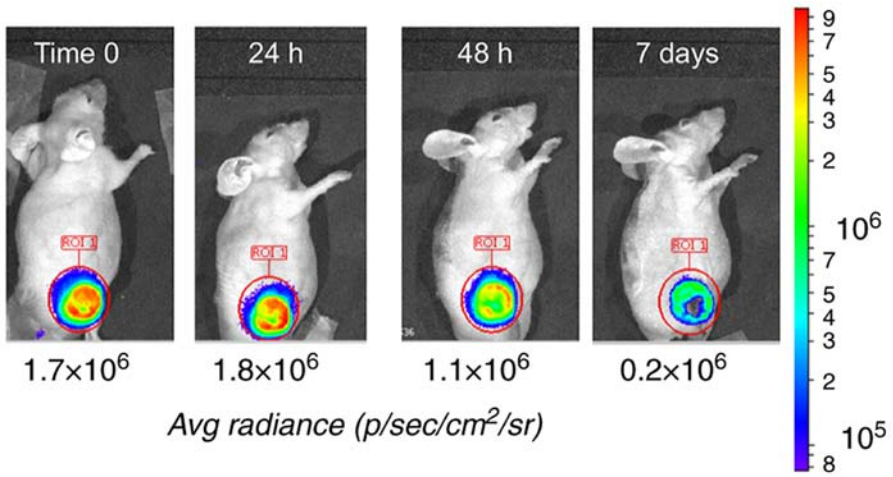

Figure 6. TOOLBOX *in vivo*. A representative mouse bearing a BRC230-cfos xenotransplant was imaged under predetermined optimal conditions (see Materials and methods), at time 0 (pre-treatment) and at the indicated times after a single treatment with the TOOLBOX cocktail (two-step protocol). Average radiance was measured in an IVIS Lumina reader after mild sedation/anaesthesia.

Discussion

Altogether, our findings provide proof-of-principle that TOOLBOX is a novel platform for antibody/drug exchange/targeting and delivery. TOOLBOX has 5 favorable features. It is a) specific, b) potent, c) modular, d) flexible, and e) conditional.

Five favorable TOOLBOX features. Specificity (a) was demonstrated by an ERBB2 pathway-dependent promoter-reporter system. Potency (b) was supported by i) the cytotoxic effects of two classes of anticancer drugs (DNA nanobinders and DM1) at their respective optimal dosages (micromolar and nanomolar), and ii) activity on ERBB2-low/non-amplified TNBC cells, e.g. a molecular breast cancer subtype that does not benefit from trastuzumab application (7), but was very recently shown to benefit from other ADCs (17). Modularity (c) was supported by the accommodation of different drug classes in TOOLBOX. Flexibility (d) in nanostructure assembly was supported by the 2-step and 3-step options, although the 2-step

protocol (ScFv + pre-formed Strep-Tactin:Strep-Tagged drug nano-assemblies) performed best in both flow cytometry and drugging experiments (Fig. S2). This is not surprising, since a second-order reaction is much more likely to occur than a third-order reaction. Additionally, the 2-step protocol requires a single 1 h interval between the two i.v. administrations of ScFv and the preformed TOOLBOX nano-complex (Fig. 6), which makes it more convenient than the 3-step protocol, particularly *in vivo*.

However, the most favorable built-in feature of TOOLBOX is conditional reactivation (e) of NAXT and DM1T compounds, e.g. the restoration of cytotoxic properties upon funneling into TOOLBOX-mediated cellular dispatch. The mechanism is being investigated. We favor the possibility that ERBB2:TOOLBOX complexes are engulfed through the default endocytic receptor internalization pathway, and then intracellular release of the active drugs, possibly through cleavage/degradation of the Strep-Tag. This route may be precluded to tagged prodrugs alone.

TOOLBOX technical limitations. Challenges were encountered of five types: Tagging, stoichiometry, nano-assembly formation, Strep-Tactin valence, and payload selection/adaption. Although there were tag position preferences, Strep-Tagging was possible at both ScFv ends with little if any interference with the small antigen-binding ScFv domain. Production is underway of Strep-Tagged, humanized, whole IgGs. Stoichiometry of the TOOLBOX building blocks, their order of addition, and the identification of the right valence of the Strep-Tactin adapter required careful troubleshooting. The critical step was the development of a Strep-Tactin polymer with a high nominal valence, to support simultaneous binding to tagged ScFv and drugs. Improved Strep-Tactin scaffolds will have to be created for controlled, optimized oligomerization.

The last and most crucial TOOLBOX step was Strep-Tagging of small drugs. The process was rather inefficient (1 good candidate out of 10 screened) with berberine derivatives. Building on this, we switched to DM1, that is a preferred ADC payload. An appropriate linker-spacer was incorporated with the aim of spatially separating the cytotoxic DM1 moiety from the polypeptide tag. The first compound generated by this strategy was found to work as intended.

Strep-Tactin nano-assemblies, that are the core adapters of all our TOOLBOX drugs, are estimated to be ~9 nm in size, e.g. smaller than most drug-entrapping systems such as liposomes, synthetic membranes, and protein nanocages (18). This may favor tissue penetration, but drug delivery is expected to be lesser than in systems based on massive drug entrapment.

Altogether, the extensive trouble-shooting reported herein clearly illustrates that TOOLBOX is innovative but, at the same time, it is still at the proof-of-concept stage. As compared to a conventional ADC, the use of multiple building blocks/components potentially introduces additional issues such as scaffold immunogenicity and unfavorable pharmacokinetics. These may slow down or hamper its industrial exploitation, and will have to be addressed in future studies.

TOOLBOX precedents. TOOLBOX bears some analogies with an avidin-biotin T lymphocyte redirection system independently proposed long time ago by two groups (19,20). These authors took advantage of biotin-labelled antitumor antibodies, HLA class I tetramers refolded around strong viral peptide antigens, and avidin, to re-direct virus-specific T cells toward tumor cells. TOOLBOX can similarly redirect HLA-I tetramers (e.g. HLA-A2 Streptamers, see Fig. 3) and antigen-specific cytotoxic T lymphocytes (data not shown). However, only TOOLBOX was shown to redirect anticancer drugs.

A distinct modular method, possibly more relevant to TOOLBOX, was developed by Metz *et al* using drug haptenized by digoxigenin, and engineered bispecific antibodies that bind tumor antigens with one arm, and digoxigenin-labelled doxorubicin with the other (21). However, the digoxigenin system requires extensive optimization for each bispecific antibody, by juxtaposition and grafting of two antigen-binding 'halves' onto selected Ig backbones to form combinatorial sets of dedicated bispecifics (22). This is far more complicated than the non-intrusive terminal addition of a very small tag to an affinity reagent of choice, like in TOOLBOX. Most importantly, unlike our NAXT and DM1T compounds, digoxigenin-labelled drugs are not known to undergo, to our knowledge, any conditional inactivation-reactivation process. Whereas a circulating active drug may have minor toxic effects when activity is in the micromolar range, like for doxorubicin (used in the digoxigenin system) and NAX compounds (used herein), it may entirely preclude the use of highly cytotoxic maytansinoids (DM1), since these work in the nanomolar range, and failed a number of clinical trials as free drugs in the 90s due to high-grade toxicity (1). Unlike all precedents, TOOLBOX nano-assemblies incorporate a unique, non-dispensable safe lock that protects host normal tissues even in the event of 'leakage' of the cytotoxic substance from TOOLBOX nano-assemblies.

Clinical translational significance. Precision oncology is a fast moving field. Massive parallel sequencing provides extensive catalogues of tumor vulnerabilities and expanded therapeutic opportunities. Multiple lines of targeted therapies are tolerated with fair to excellent quality of life. Standard regimens of targeted and non-targeted treatments (in sequence and in combination) are almost invariably adopted to counter primary as well as secondary drug resistance. Regulatory bodies authorize drugs for use at progressively earlier cancer stages, e.g. in the neoadjuvant and adjuvant settings (23). And yet, despite flexibility in cancer targeting is clearly perceived as a priority, the issue has not been really addressed at the biotech level due to a lack of technical solutions. To our knowledge, TOOLBOX is one of the few attempts to create a platform for the development of families of ADC-like objects with no need to manufacture many distinct ADCs. Switching and/or combining antibodies and/or anticancer agents (by TOOLBOX, its improvements, or other unrelated technologies) will make it possible to provide effective ADC-like target therapies to different patients and/or the same patient through disease stages and progression. This may effectively address some unmet needs of precision drugging.

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Availability of data and materials

W6/800 and TOOLBOX are patent pending (WO2017167967, WO2018138676) and their exploitation rights are owned by IBI Lorenzini and the TOOLBOX Consortium, respectively. Restrictions may apply to their distribution. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions

ET and MA tested the TOOLBOX concept and performed flow cytometry. LS, FC, GS and LC designed and prepared the tagged ScFv. NK and KF designed and prepared the GFP promoter-reporter construct. GH and JB designed and manufactured Strep-Tactins. ET and IM performed the animal studies. PL designed and manufactured NAX and NAXT compounds. PG conceptualized TOOLBOX and wrote the manuscript with the contribution of all authors. All authors have approved the final version of the manuscript.

Ethics approval and consent to participate

In vivo experiments were approved (prot. 665/2017-PR) by the Veterinary Section of the Italian Ministry of Health.

Patient consent for publication

Not applicable.

Competing interests

LC and JB are full-time employees of IBI Lorenzini and IBA Lifesciences, that hold exploitation rights for W6/800, Strep-Tag technologies and TOOLBOX. PG is directly supported by Ibi Lorenzini funds. All the other coauthors declare no conflict of interest.

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