Data S1.

Supplementary materials and methods

Cell lines. Cells were grown in RPMI-1640/10% bovine serum supplemented with 1% l-glutamine and 1% penicillin-streptomycin (complete medium), all from Euroclone (MI, Italy).

Recombinant DNAs, Strep-Tag technology and ScFv expression. All recombinant DNAs encoding ScFvs were produced by whole gene synthesis (GenScript), cloned into the modular pENTRY51 Stargate donor vector, shuttled into the pESG142 and pESG144 acceptor/expression vectors to generate WSHPQFEK Strep-Tagged and 6x His-tagged fusion polypeptides, and stably expressed in a secretable form in CHO-S cells (Thermo Fisher Scientific, Inc.). ScFvs were purified (>95% pure by Coomassie stain) from culture supernatants by affinity chromatography on Strep-Tactin activated Sepharose. Tagged ScFvs were tested in flow cytometry for binding to live cells using: a) antibodies to the His-tag, b) Strep-Tactin conjugated to phycoerythrin (StrepTactin-PE), and c) unconjugated Strep-Tactin followed by a recombinant Strep-Tagged green fluorescent protein (One-Strep-GFP). HLA-A2 Streptamers (tetramers of HLA class I molecules refolded around a specific peptide antigen) were also used in certain experiments. They carry Strep-Tag moieties at a c-terminal, accessible site. All the reagents for Strep-Tag technology including cDNA vectors were obtained from IBA Lifesciences.

Standard TOOLBOX flow cytometry protocol. The standard TOOLBOX protocol was carried out in either 2 or 3 steps, as noted in the text, by incubating human breast cancer cells ($5x10^5$) on ice (successive incubations of 30 min each) with the same standard concentrations of the TOOLBOX components: i) tagged ScFv (0.5 μg); ii) standard phycoerythrin-conjugated Strep-Tactin (Strept-Tactin-PE) multimerized at an average theoretical multiplicity of 4 (0.75 μg); and iii) Strep-Tagged green fluorescent protein (One-Strep-GFP; 0.75 μg). In certain experiments, Strept-Tactin-PE was replaced by high multiplicity phycoerithrin-conjugated Strep-Tactin (Strept-Tactin-Mult-PE) multimerized at an average theoretical multiplicity of 10 (0.75 μg). All reagents were diluted in 50 μl of phosphate (0.01 M) buffered (pH 7.4) saline (0.9%), i.e. PBS, yielding the following final concentrations: ScFv 10 mg/ml; Strep-Tactin and One-Strep-GFP 15 μg/ml. At the end of each incubation step, the cells were washed twice with PBS. Stained cells were analyzed by two-color flow cytometry in a FACScan (BD Biosciences). In the 3-step and 2-step protocols the concentrations of the reagents were identical, but Strep-Tactin and One-Strep-GFP were preliminarily admixed in the 2-step protocol.

TOOLBOX treatment with NAX compounds. The antiproliferative effects of TOOLBOX were assessed by [^{3}H]-Thymidine incorporation. Subconfluent SK-BR-3 cells were incubated for 30 min at 4˚C in 96-well plates containing 100 μl of complete medium supplemented with a) Strep-Tagged ScFv or b) its inactive CDR mutant ScFv-mut4 (both at 10 μg/ml), or c) a NAX compound, or d) its corresponding Strep-Tagged NAX derivative (NAXT). Additional microwells received ScFv as above, and e) pre-formed TOOLBOX complexes prepared by incubating for 45 min the same amounts of NAXT and Strep-Tactin-Mult (0.75 μg/well). At the end of the 4˚C incubation, all final microwell volumes were brought to 200 μl with complete medium, moved to a CO_2 incubator, and the cells were grown for 72 h. Proliferation was assessed by measuring ^{3}[H]-Thymidine incorporation during the last 4 h of incubation. All determinations were in triplicate.

ERBB2 pathway-dependent promoter-reporter luciferase assay and TOOLBOX treatment with DM1T. To visualize and quantitate ERBB2 signaling *in vitro* and *in vivo* (in mice), a 756-bp fragment from the c-fos promoter containing three c-fos binding sites (1) was *Eco*RI/*Bgl*II cloned in pNL 1.1 (Promega Corp.) upstream of a reporter gene encoding an improved NanoLuc® that emits 150 times more intense light than the luciferase protein. The construct was stably transfected into SK-BR-3 and BRC230 breast carcinoma cells using the Lipofectamine 3,000 Transfection Reagent (Thermo Fisher Scientific, Inc.) according to the protocol provided by the supplier. The c-fos promoter was chosen to drive the expression of luciferase since c-fos is a major downstream sensor of receptor tyrosine kinase (RTK) activity. To test c-fos activity, aliquots of $5x10^4$ cells were either left untreated or stimulated with varying concentrations of human epidermal growth factor (EGF; ImmunoTools) for 16 h at 37˚C. Cells were then harvested and lysed by 3 min incubation in 100 μl of Nano-Glo® Luciferase Assay Buffer (Promega Corp.) containing the furimazine substrate (commercially provided in solution at optimal concentrations). Twenty microliters were transferred to luminometer-grade 96-well-plates, and bio-luminescence was directly measured in a Promega luminometer as previously described (2). For TOOLBOX treatment, cells were pre-incubated with TOOLBOX components for 45 min at 4˚C exactly as in the case of NAX compounds, moved to a CO_2 incubator, and grown for 72 h, and then lysed in the presence of the furimazine substrate, and finally assessed for bio-luminescence in a luminometer (2).

Animal experiments. *In vivo* experiments were approved (prot. 665/2017-PR) by the Veterinary Section of the Italian Ministry of Health. BRC230-cfos cells were inoculated subcutaneously in 6-week-old nu/CD1 female (n=6) mice (Charles River Laboratories, Calco, Italy) and grown to a tumor size of up to 1,000 mm^3. Mice were housed in a fully certified facility, under controlled temperature and atmosphere (22±2˚C; 50-60% humidity). Forced ventilation (15 changes/h), a circadian dark/light (14/10 h) cycle, and free access to fresh food and water were insured by at least twice-a-day inspections. Mice were treated by tail vein (i.v.) injection of ScFv (or ScFvmut4) at 10 mg/kg, followed 1 h later by a pre-mix of Strep-Tactin-Mult (7.5 mg) and DM1T (2 mg/kg). At two or more of selected time points (time 0, day 1, day 2, day 7) mice were lightly anesthetized by intramuscular injection of Zoletil® (0.8 ml/kg), then the substrate furimazine (5 μg in 100 μl) was i.v. injected, as previously described (3). Mice were sacrificed by cervical dislocation after profound anesthesia by Zoletil® at the above dosage plus Rompun (1 mg/kg). The light emitted by the NanoLuc reporter was imaged in a IVIS Lumina (Perkin Elmer, Inc.), and peaked at 5 min, remaining stable for at least 30 min. All images were taken at 5 min, with a pre-set camera exposure of 1 min. Gating and quantitation of light emission was automatically carried out by on-board Lumina software (Living Image 4.3.1).

References

1. Schonthal A, Herrlich P, Rahmsdorf HJ and Ponta H: Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 54: 325-334, 1988.
2. Knutti N, Kuepper M and Friedrich K: Soluble extracellular matrix metalloproteinase inducer (EMMPRIN, EMN) regulates cancer-related cellular functions by homotypic interactions with surface CD147. FEBS J 282: 4187-4200, 2015.
3. Stacer AC, Nyati S, Moudgil P, Iyengar R, Luker KE, Rehemtulla A and Luker GD: NanoLuc reporter for dual luciferase imaging in living animals. Mol Imaging 12: 1-13, 2013.
4. Pierpaoli E, Fiorillo G, Lombardi P, Salvatore C, Geroni C, Piacenza F and Provinciali M: Antitumor activity of NAX060: A novel semisynthetic berberine derivative in breast cancer cells. Biofactors 44: 443-452, 2018.

Figure S1. Strep-Tactin-PE does not support the TOOLBOX protocol. Two-color flow cytometry of SK-BR-3 cells incubated in the 2-step TOOLBOX protocol with ScFv and one-Strep-GFP at the standard concentrations (see above) of 0.5 and 0.75 μg per well, respectively. Strep-Tactin-PE:One-Strep-GFP complexes were formed using the standard One-Strep-GFP concentration and the indicated, increasing Strep-Tactin-PE concentrations. (A) Negative control in which one crucial TOOLBOX ingredient is omitted. (B) Reference red fluorescence (binding of Strep-Tactin-PE). (C-F) As in B, but with scalar amounts of Strep-Tactin-PE.

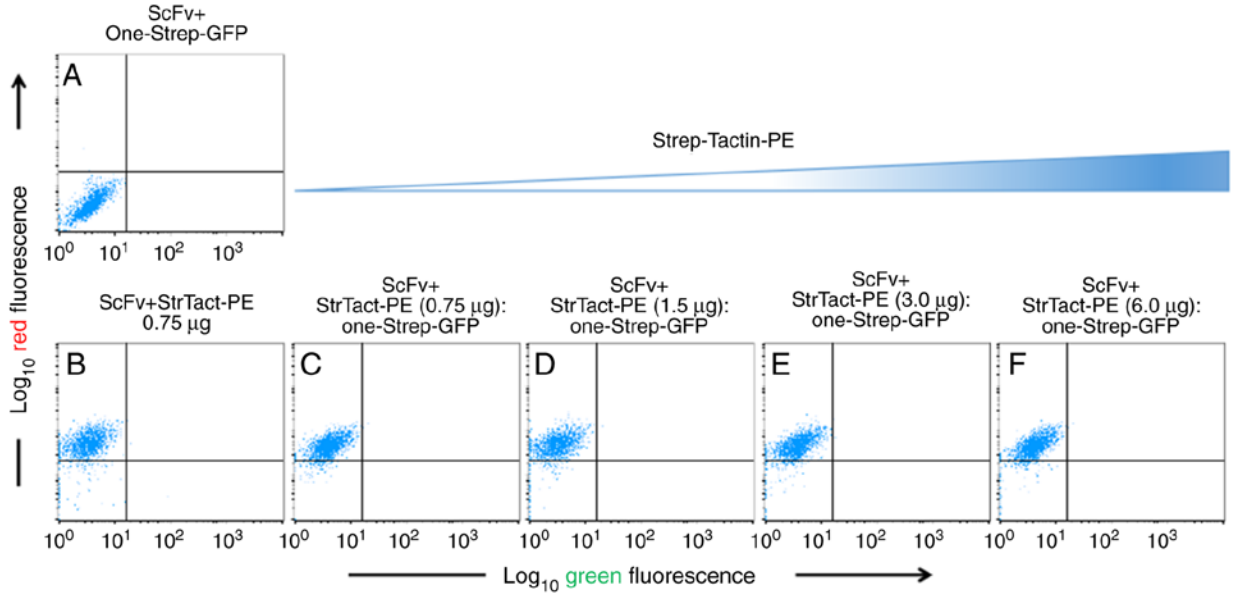

Figure S2. Paradoxical inhibition of Strep-Tactin-PE binding by excess One-Strep-GFP. Two-color flow cytometry of SK-BR-3 cells incubated in the two-step (A-D) and 3-step (E-H) TOOLBOX protocols with ScFv (10 μg/ml), Strep-Tactin-PE (0.75 μg), and the indicated, decreasing amounts of One-Strep-GFP. Note the more rapid rescue of red fluorescence in the 3-step (F-H) as compared with the 2-step (B-D) protocol.

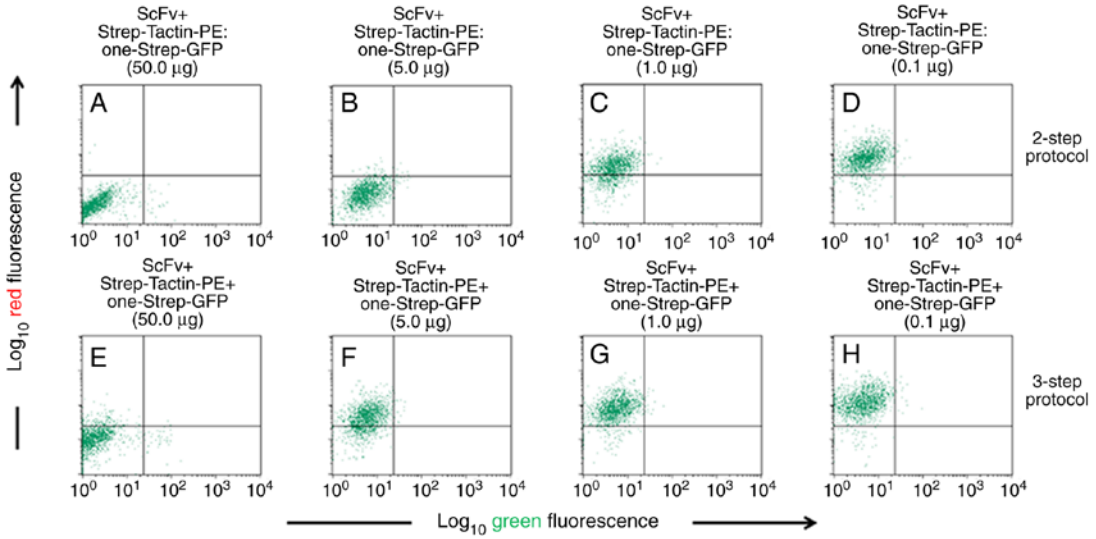

Figure S3. Comparison between Strep-Tactin-PE and Strep-Tactin-Mult-PE. Two-color flow cytometry of SK-BR-3 cells incubated with either (A) Strep-Tact-Mult-PE only (control) or standard amounts of the TOOLBOX ingredients: ScFv at 0.5 μg and either Strep-Tactin-PE (B) or Strep-Tactin-Mult-PE (C), both at 0.75 μg. Although no green fluorescent reagent are provided, a 2d fluorescence plot is shown to facilitate comparison with the results presented in the other figures.

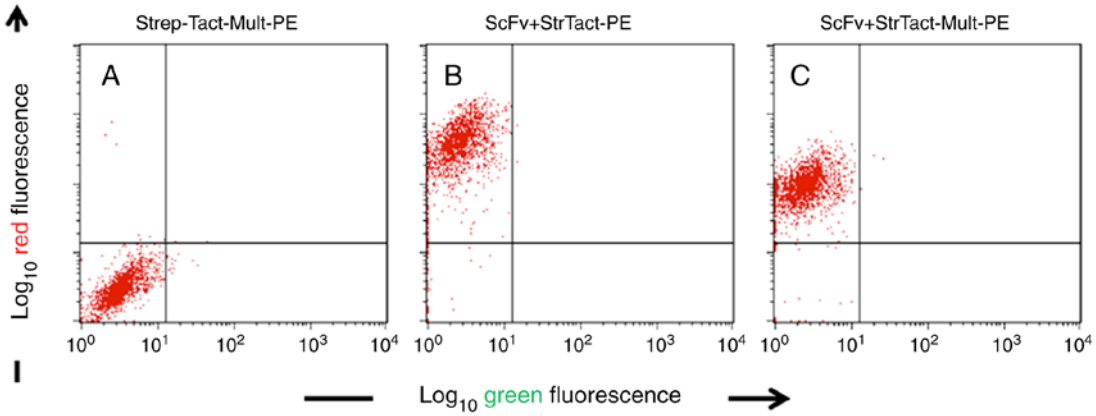

Figure S4. TOOLBOX cocktail with unlabelled Strep-Tactin-Mult. Two-color flow cytometry of SK-BR-3 cells incubated with standard amounts of the TOOLBOX ingredients: ScFv at 0.5 μg; Strep-Tactin-PE, unlabelled Strep-Tactin-Mult, and Strep-Tactin-Mult-PE all at 0.75 μg; one-Strep-GFP at 0.75 μg. (A and B) Negative controls obtained by omitting one or more crucial ingredients. (C) Reference Strep-Tactin-Mult-PE binding; (D) One-Strep-GFP binding after fluorescent-labelled Strep-Tactin-PE has been replaced with the same amounts of its unlabelled counterpart.

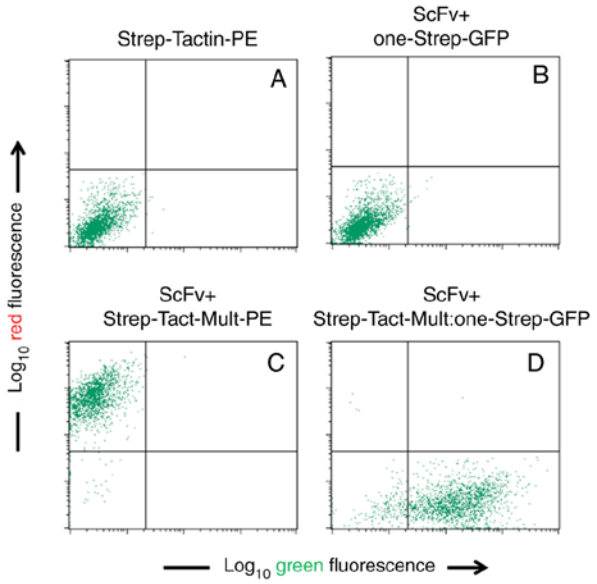

Figure S5. Organic synthesis of NAX compounds. The strategy is outlined to generate the four final NAX compounds (names in red). The parent compounds were the previously described berberine and NAX 014 compounds (boxed). The diagram outlines major steps, chemicals and conditions.

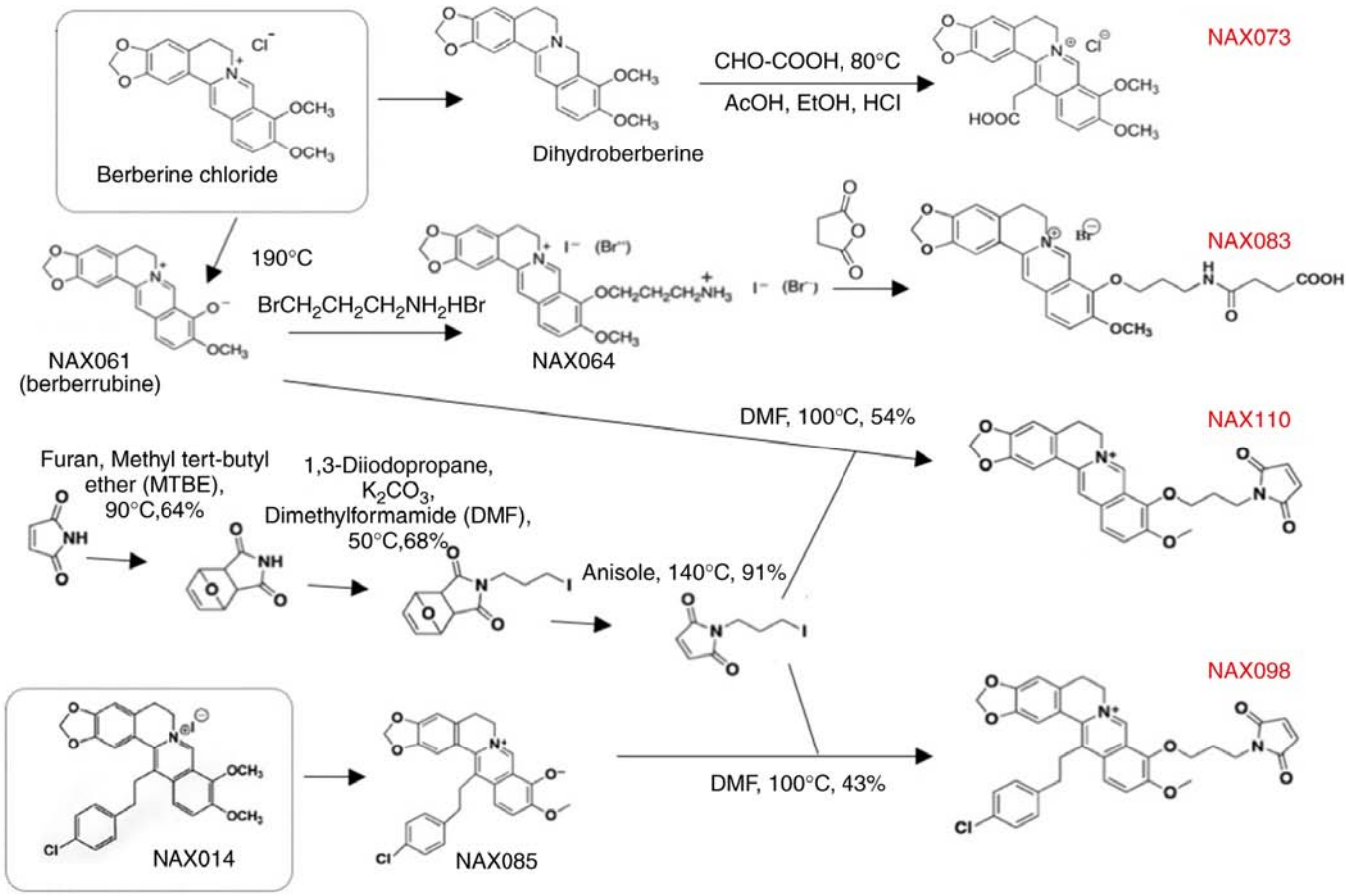

Figure S6. Structure of Strep-Tagged DNA nanobinders. The four selected berberine derivatives and their Strep-Tagged variants (NAX and NAXT, respectively) are displayed.

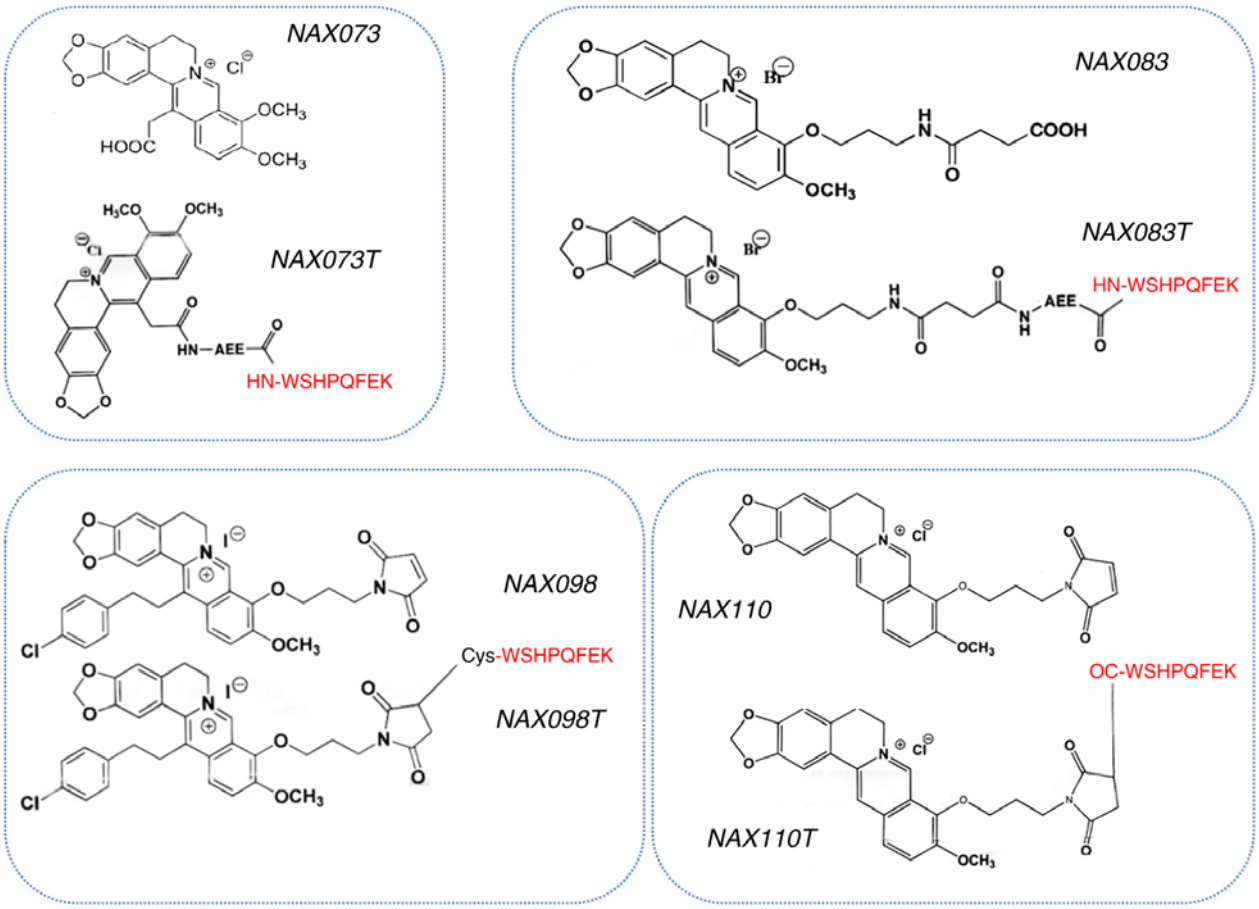

Figure S7. Anti-proliferative effects of NAX and NAXT compounds. [^{3}H]-Thymidine incorporation was assessed over a 4-h radioactive pulse at the end of a 72-h 2-step treatment of SK-BR-3 cells with the TOOLBOX cocktail. Cells were grown in the presence of ScFv in the first step, followed in the second step by each of the 4 indicated NAXT compounds pre-mixed at the indicated concentrations with Strep-Tactin-Mult (orange bars), as described above in Data S1. Controls included single-reagent incubations with ScFv144, its inactive CDR mutant, as well as each NAX compound and its tagged NAXT counterpart (grey, black, yellow and blue bars, respectively). Results are expressed as percent of radionuclide incorporation compared to the untreated (complete medium) cells. Out of 4 novel NAX compounds, at least 2 (NAX098 and NAX110) displayed the anti-proliferative activity typical (4) of this class of nanobinders (compare black and yellow bars), while Strep-Tag addition resulted in complete or nearly complete inactivation (compare yellow and blue bars, e.g. NAX098 to NAX098T and NAX110 to NAX110T). However, and unexpectedly, anti-proliferative activity was surprisingly reinstated when NAXT compounds were administered by the TOOLBOX protocol (orange bars). Reinstatement was proportional to the original activity of the parental compound in both the active NAX/NAXT pairs (compare yellow and orange bars). NAX098T results (boxed) are also displayed in Fig. 4. This experiment was carried out three times with similar results. $^{**}p<0.01$ compared to no drugs.

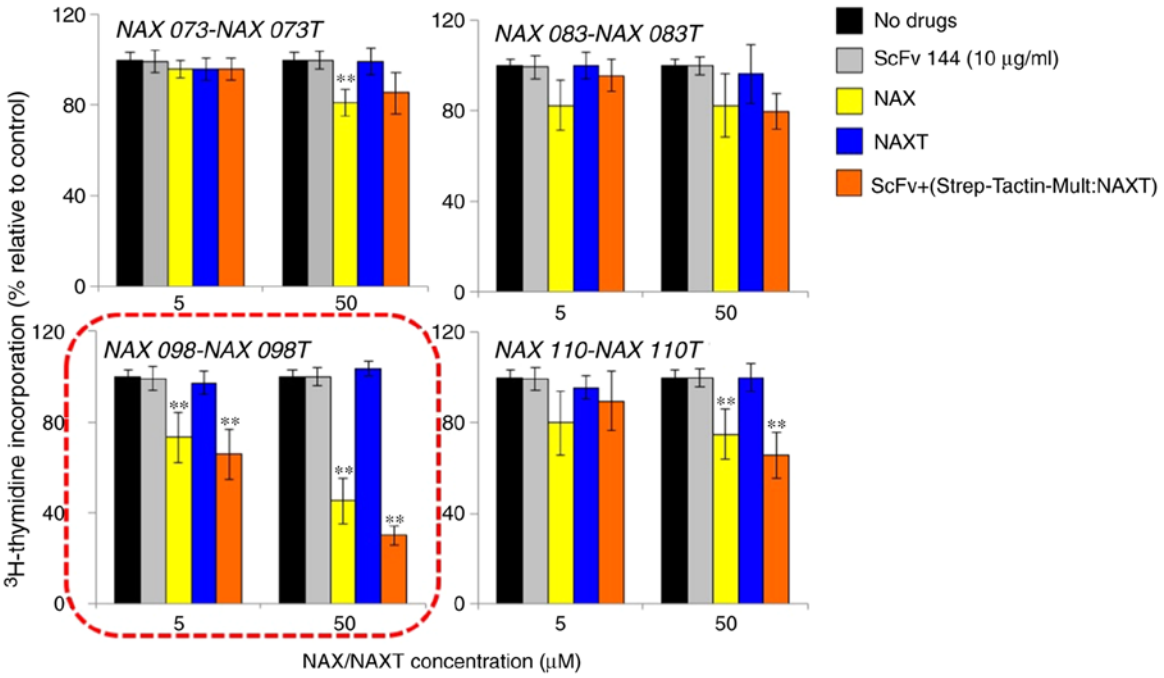

Figure S8. EGFR and ERBB2 expression of BRC230 cells. Flow cytometry comparison of ERBB2-amplified/overexpressed SK-BR-3 and ERBB2-low/TNBC BRC230 breast cancer cells. Antibodies were used at 10 μg/ml (30-min incubation), followed by FITC-labelled antimurine Ig (30 min), exactly as in Fig. 1. EGFR, epidermal growth factor receptor; TNBC, triple-negative breast cancer.

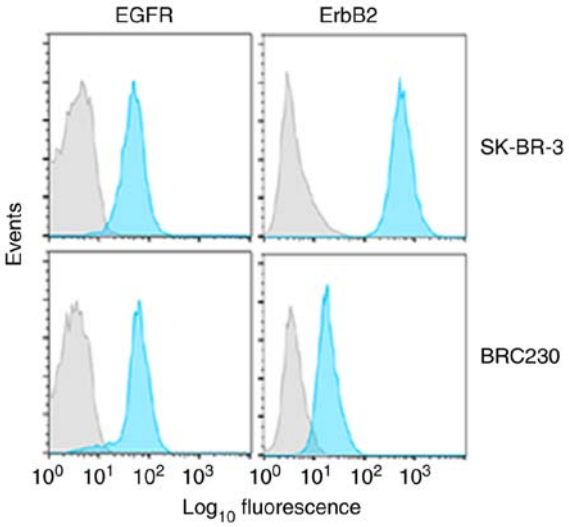

Figure S9. EGF-dependent luciferase reporter gene activation in SK-BR-3 and BRC230 cells stably transfected with a c-fos promoter-driven NanoLucR reporter construct. SK-BR-3-cfos (A) and BRC230-cfos (B) cells ($5x10^4$/well) carrying the c-fos promoter-NanoLuc reporter construct were treated for 16 h with the indicated EGF concentrations, and lysed in 100 μl of Nano-Glo Luciferase Assay Buffer (Promega Corp.) containing the enzyme substrate. Light emission (relative luminescence units; RLU) are the means ± SD of three independent experiments. Treatment conditions are more thoroughly described in Data S1. EGF, epidermal growth factor.

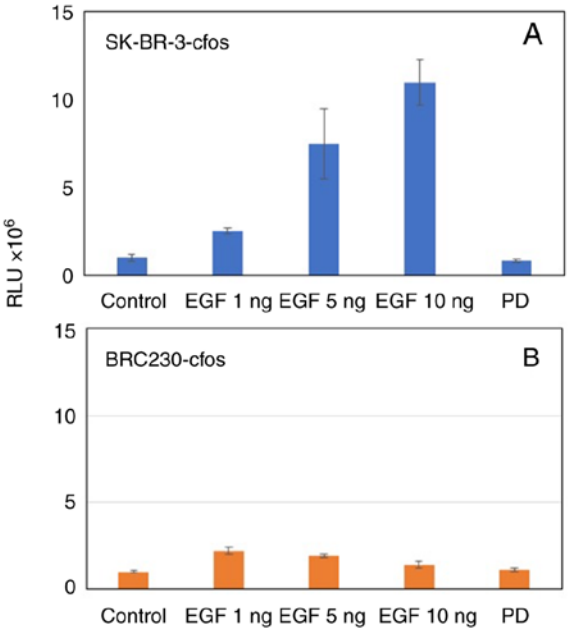

Table SI. CDR sequence homology and ScFv binding in flow cytometry.

	% Conserved	MFI (10 μg/ml)[a]	% Binding	MFI (2 μg/ml)[a]	% Binding
ScFv WT	100	249	100	154	100
Scfv mut 1	41	175	62	15	10
ScFv mut 2	15	36	15	9	6
ScFv mut 3	2	19	8	0	0
ScFv mut 4	0	0	0	0	0

CDR, complementarity determining regions; MFI, mean fluorescence intensity; WT, wild-type; mut, mutant. [a]After subtraction of average flow cytometry background with isotype-matched Ig (MFI=7.0). Data are from Fig. 2.