

Figure S1 – Assessment of the HER2 status and localization. The HER2 status in the gastric cancer cell lines MKN1, MKN7 and NCI-N87 was evaluated by IHC. The expression of the receptor was analyzed using the HER2-specific antibody 4B5. The staining of HER2 in the cell lines NCI-N87 and MKN7 is positive (score 3+), whereas it is negative (score 0) for MKN1. HER2 is mainly but not exclusively located at the plasma membrane. Pictures were recorded with a 40 x resolution.

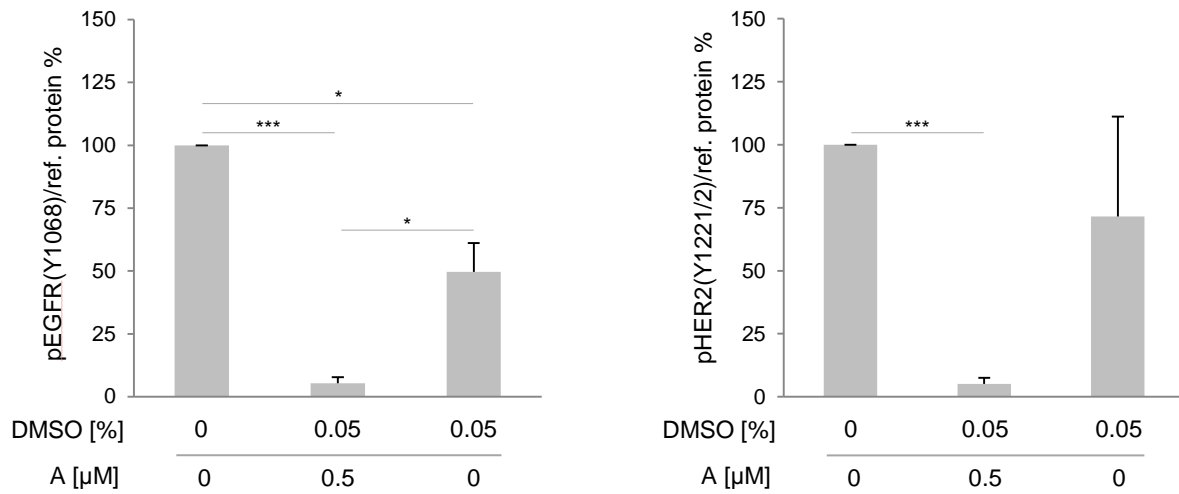


Figure S2 - Effects of DMSO on the activation of EGFR and HER2 in NCI-N87 cells.

Protein activation was detected in total lysates of DMSO and afatinib treated NCI-N87 cells by Western blotting using phospho-specific antibodies directed against (a) pEGFR (Y1068) and (b) pHER2 (Y1221/2). A significant (pEGFR) and slight (pHER2) effect of DMSO was detected, but decrease in activation of EGFR and HER2 in afatinib-treated NCI-N87 cells was more pronounced compared to DMSO-controls, thus the detected effects result from afatinib. The mean value of at least three independent experiments is shown. Significant effects are indicated by $*0.01 < p\text{-value} \leq 0.05$ or $***\leq 0.001$ (one-sample t-test). Abbreviation: A = afatinib

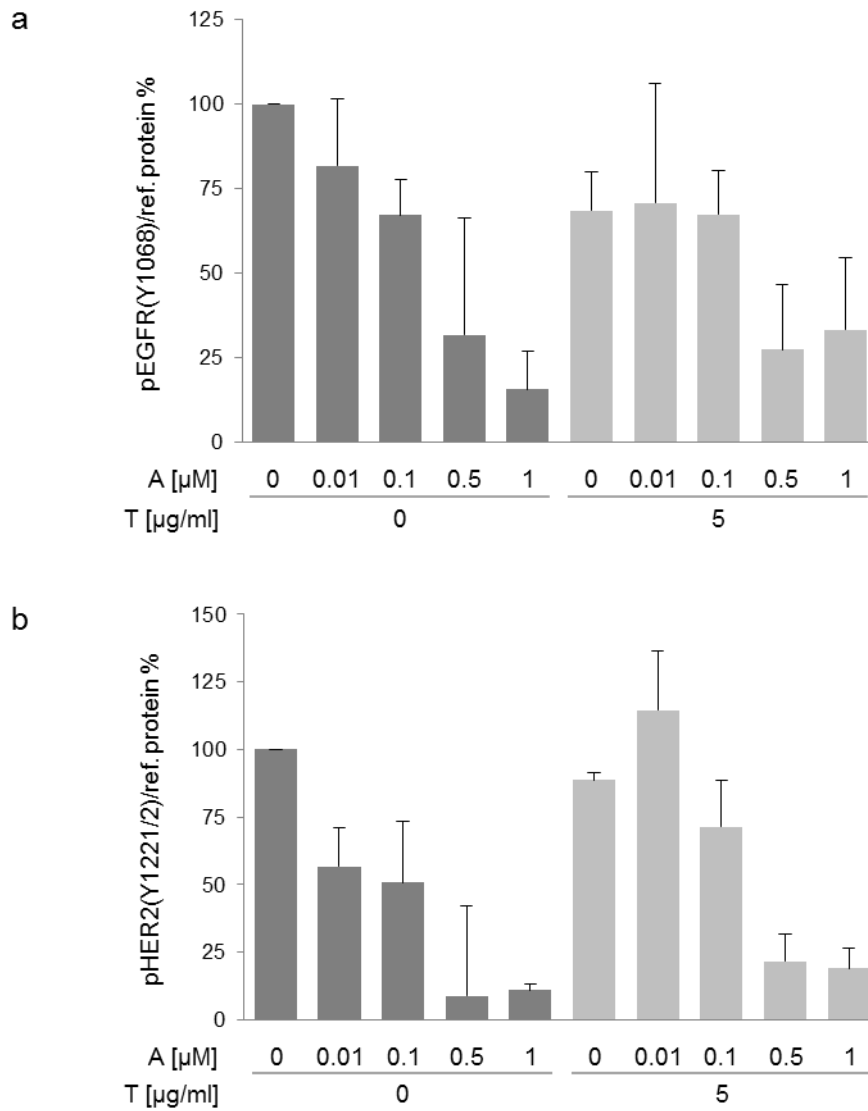


Figure S3 - Concentration-dependent effect of trastuzumab and afatinib on the activation of EGFR and HER2 in NCI-N87 cells. The levels of activated receptors were determined after 5 minute treatment of NCI-N87 cells with trastuzumab (5 $\mu\text{g/ml}$) and/or afatinib (0.01, 0.1, 0.5, 1 μM) by Western blot analysis with pEGFR-specific (Y1068) and pHER2-specific (Y1221/2) antibodies. The average phosphorylation levels were quantified using densitometric analysis and calculated in relation to the levels of the reference proteins α -tubulin and β -actin (+SD). The mean value of at least three independent experiments is shown. Statistical p-values are listed in Table S1. Abbreviations: A = afatinib, T = trastuzumab

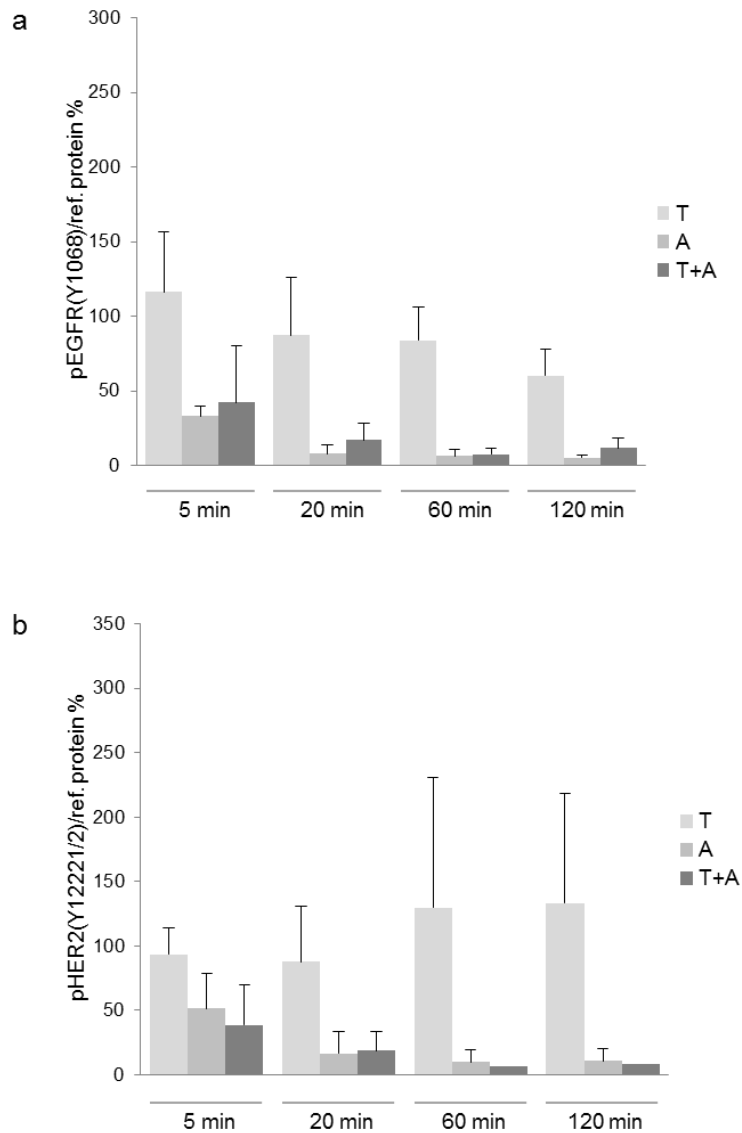


Figure S4 - Kinetic effect of trastuzumab and afatinib on the activation of EGFR and HER2 in NCI-N87 cells. The levels of activated receptors were determined after treatment of NCI-N87 cells with trastuzumab (5 μ g/ml) and/or afatinib (0.5 μ M) by Western blot analysis. Cells were treated for 5, 20, 60 and 120 min. Activation of the receptors were analyzed using pEGFR-specific (Y1068) and pHER2-specific (Y1221/2) antibodies. The average phosphorylation levels were quantified using densitometric analysis and calculated in relation to the levels of the reference proteins α -tubulin and β -actin (+SD). The mean value of at least three independent experiments is shown. Statistical p-values are listed in Table S1. Abbreviations: A = afatinib, T = trastuzumab

a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	+																							+
B	EGFR	HER2	HER3	HER4	FGF R1	FGF R2 α	FGF R3	FGF R4	Insulin R	IGF-1R	AXL	DTK												
C	MER	HGF R	MSP R	PDGF R α	PDGF R β	SCF R	Flt-3	M-CSF R	C-Ret	ROR1	ROR2	Tie-1												
D	Tie-2	TrkA	TrkB	TrkC	VEGF R1	VEGF R2	VEGF R3	MuSK	Eph A1	Eph A2	Eph A3	Eph A4												
E	Eph A6	Eph A7	Eph B1	Eph B2	Eph B4	Eph B6	ALK	DDR1	DDR2	Eph A5	Eph A10													
F	+		Eph B3	RYK																				PBS

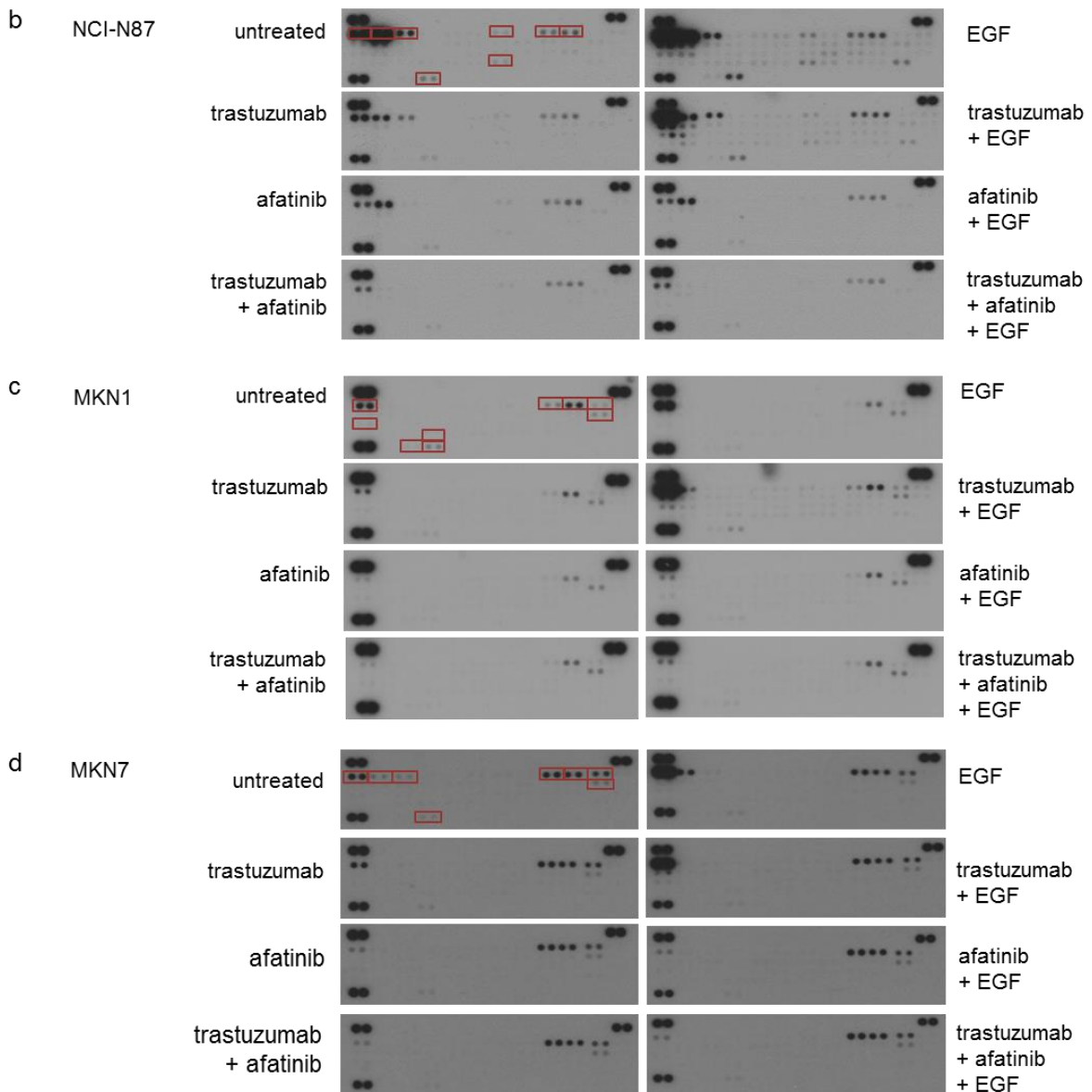


Figure S5 - Receptor tyrosine kinases analyzed by the RTK proteome profiler. (a) Mapped receptor tyrosine kinases on the membrane of the RTK proteome profiler and the entirety of activated proteins (red squares) in the investigated cell lines (+, positive control; PBS, negative control). (b)-(c) Cells were treated for 20 min with 5 μ g/ml trastuzumab, 0.5 μ M afatinib and/or 5 ng/ml EGF, respectively. Activated, and under different conditions

regulated proteins were detected with chemiluminescence. Varied proteins were activated in the cell line (b) NCI-N87, (c) MKN1 and (d) MKN7 indicated by red squares. The depicted results are representative of three independent experiments.

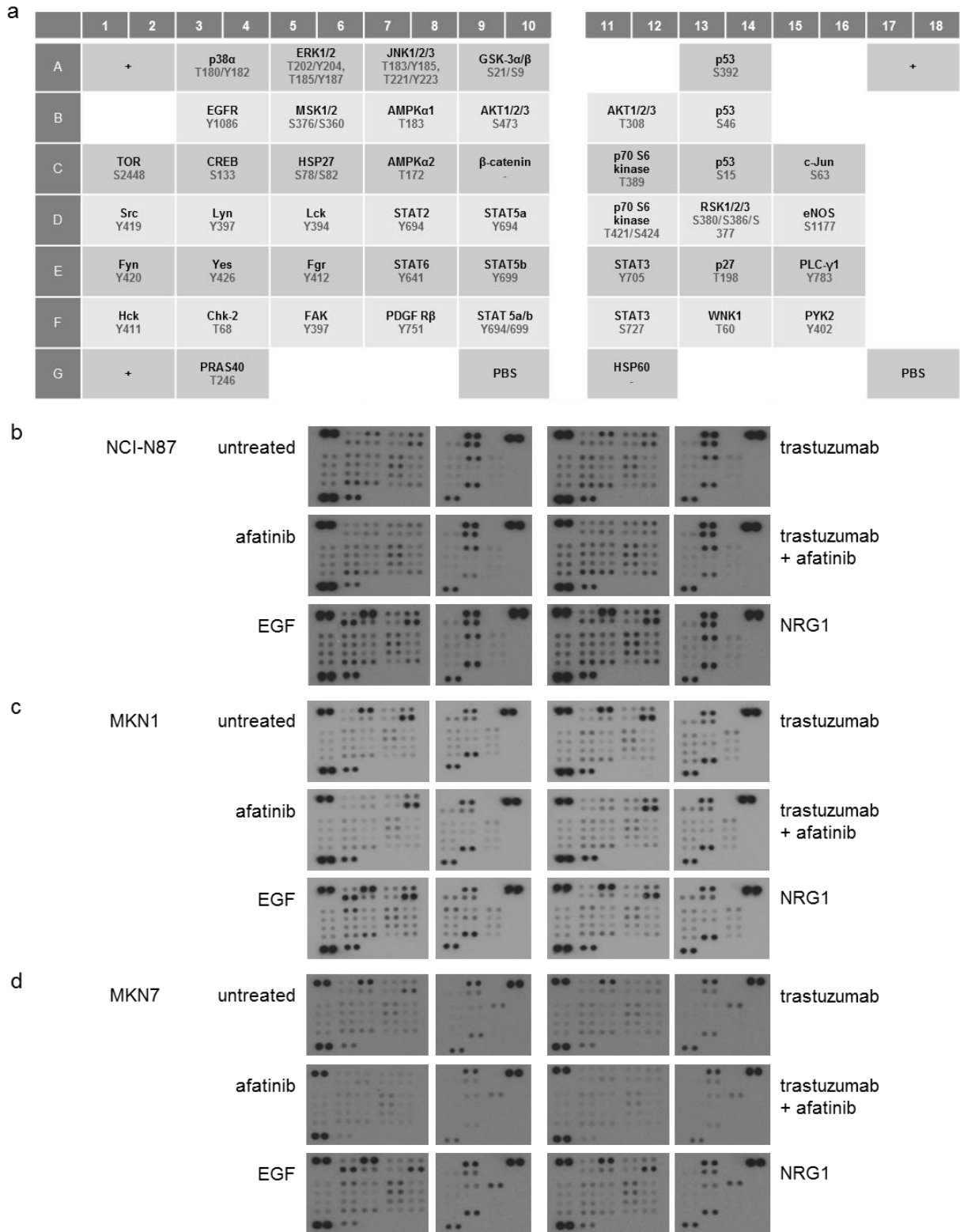


Figure S6 - Phosphorylated kinases analyzed by the kinase proteome profiler. (a) Arrangement of the kinases in the kinase proteome profiler assay, indicating the specific phosphorylation sites. (b)-(c) Cells were treated for 20 min with 5 μg/ml trastuzumab and/or 0.5 μM afatinib or for 5 min with 5ng/ml EGF or 20 ng/ml NRG1, respectively. The depicted results are representative of three independent experiments of the cell lines NCI-N87 (b), MKN1 (c) and MKN7 (d).

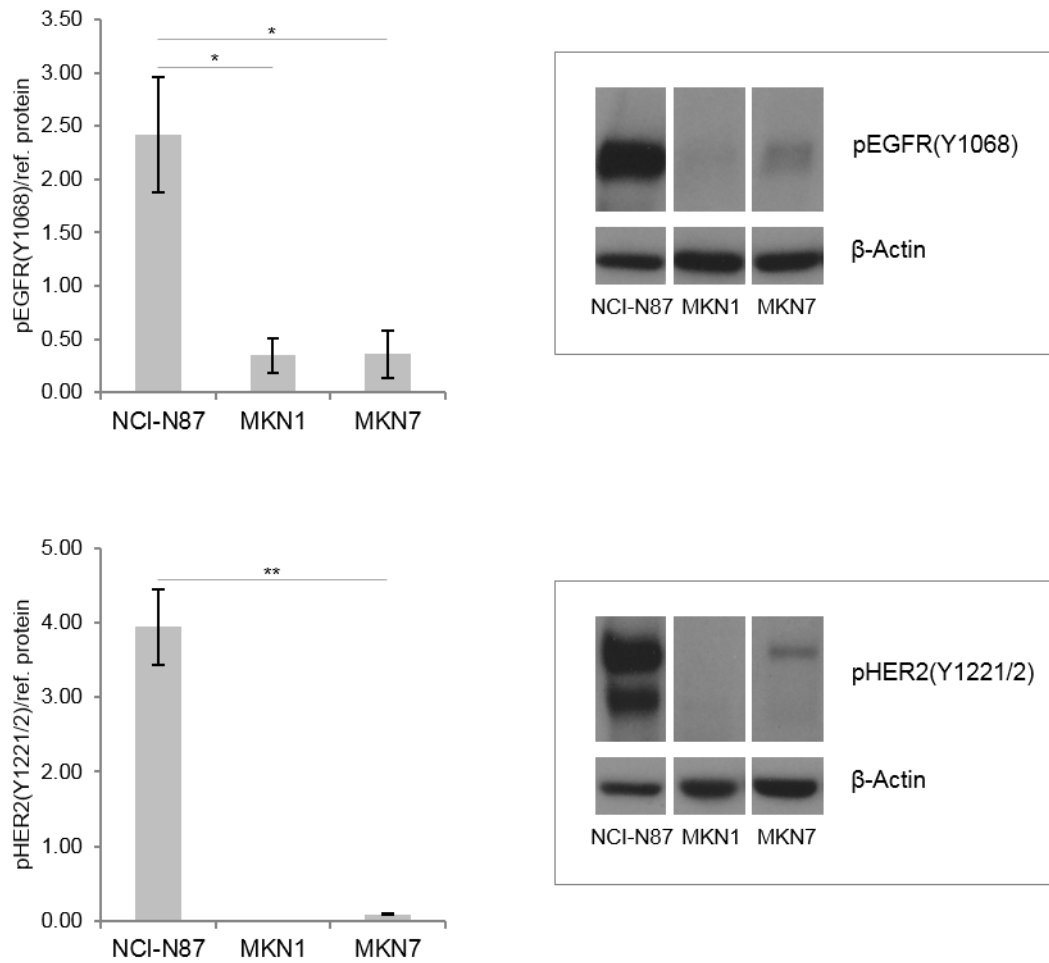


Figure S7 – Comparison of EGFR and HER2 activation in NCI-N87, MKN1 and MKN7 cells. Protein activation was detected in total lysates of untreated cells by Western blotting using phospho-specific antibodies directed against (a) pEGFR (Y1068) and (b) pHER2 (Y1221/2). The average phosphorylation levels were quantified using densitometric analysis and calculated in relation to the levels of the reference proteins α -tubulin and β -actin (+SD). The three different cell lines were detected on one membrane for direct comparison. White lines indicate samples which were cut out since they are not important in this context. Significant effects are indicated by * $0.01 < p\text{-value} \leq 0.05$ or ** $0.001 < p\text{-value} \leq 0.01$ (Two-sided Welch t-test). No statistical analysis was performed for the activation of HER2 in MKN1 because signals were below detection limits.

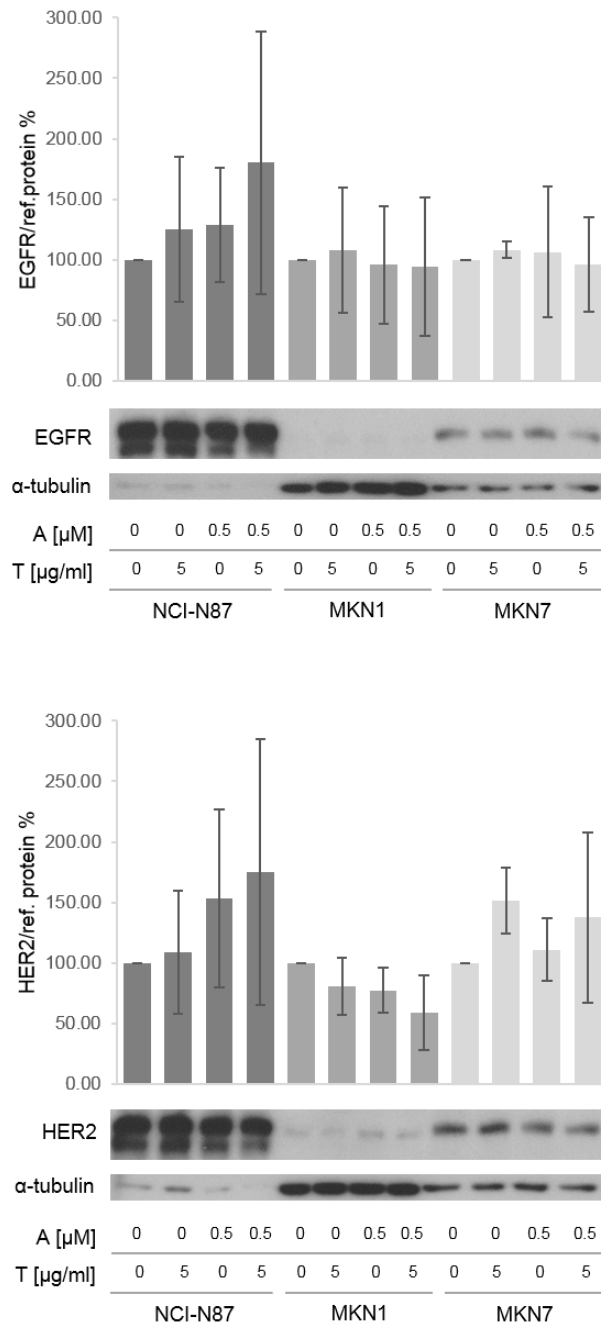


Figure S8 – Effect of trastuzumab and afatinib on the expression of EGFR and HER2 in NCI-N87, MKN1 and MKN7 cells. Protein expression was detected in total lysates by Western blotting using specific antibodies directed against (a) EGFR and (b) HER2. The same lysates were used as generated for the RTK Proteome Profiler. The average abundance levels were quantified using densitometric analysis and calculated in relation to the levels of the reference proteins α -tubulin (+SD). The three different cell lines were detected on one membrane for direct comparison. Quantitative signals are only comparable within one cell line. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the different treatments. One samples t-test was used to test for statistical significant differences.

Table S1 - Pairwise comparisons of EGFR and HER2 activation in NCI-N87 cells (treatment and control). Cells were treated with afatinib or a corresponding amount of DMSO as control. Two-sided Welch t-test and one-sample t-test were used. Significant p-values are shown. Abbreviations: Ctrl: Control (untreated); A: 0.5 μ M afatinib, DMSO: 0.05% DMSO

Activation of protein	Groups (X vs. Y)		Mean \pm SD (X)	Mean \pm SD (Y)	p-value
	X	Y			
EGFR	Ctrl	A	100	5.280 \pm 2.503	< 0.001
		DMSO	100	49.697 \pm 11.368	0.017
	A	DMSO	5.280 \pm 2.503	49.697 \pm 11.368	0.017
HER2	Ctrl	A	100	5.160 \pm 2.380	< 0.001

Table S2 - Pairwise comparisons of EGFR, MAPK and AKT activation in NCI-N87 cells between various treatments. Cells were treated with 5 µg/ml trastuzumab and/or different concentrations of afatinib. Western blot analyses were done in biological replicates. A two-sided Welch t-test and a one-sample t-test were used. Significant p-values are shown, relevant comparisons are highlighted in bold font. Abbreviations: Ctrl: Control (untreated); T: 5 µg/ml trastuzumab; A 0.01: 0.01 µM afatinib; A 0.1: 0.1 µM afatinib; A 0.5: 0.5 µM afatinib; A 1: 1 µM afatinib

Activation of protein	Groups (X vs. Y)		Mean ± SD (X)	Mean ± SD (Y)	p-value
	X	Y			
EGFR	Ctrl	A 0.1	100	67.183 ± 10.495	0.032
	Ctrl	A 1	100	15.766 ± 11.208	0.006
	Ctrl	T	100	68.626 ± 11.529	0.042
	Ctrl	T+A 0.1	100	67.487 ± 13.089	0.050
	Ctrl	T+A 0.5	100	27.565 ± 19.116	0.022
	Ctrl	T+A 1	100	33.342 ± 21.202	0.032
	A 0.01	A 1	81.808 ± 19.764	15.766 ± 11.208	0.013
	A 0.01	T+A 0.5	81.808 ± 19.764	27.565 ± 19.116	0.027
	A 0.01	T+A 1	81.808 ± 19.764	33.342 ± 21.202	0.045
	A 0.1	A 1	67.183 ± 10.495	15.766 ± 11.208	0.004
	A 0.1	T+A 0.5	67.183 ± 10.495	27.565 ± 19.116	0.049
	A 1	T	15.766 ± 11.208	68.626 ± 11.529	0.005
	A 1	T+A 0.1	15.766 ± 11.208	67.487 ± 13.089	0.007
	T	T+A 0.5	68.626 ± 11.529	27.565 ± 19.116	0.044
	T+A 0.1	T+A 0.5	67.487 ± 13.089	27.565 ± 19.116	0.047
HER2	Ctrl	A 1	100	10.081 ± 2.085	0.000
	Ctrl	T	100	88.465 ± 2.556	0.016
	Ctrl	T+A 0.5	100	22.512 ± 9.852	0.005
	Ctrl	T+A1	100	21.163 ± 7.513	0.003
	A 0.01	A 1	65.003 ± 14.329	10.081 ± 2.085	0.020
	A 0.01	T+A 0.5	65.003 ± 14.329	22.512 ± 9.852	0.017
	A 0.01	T+A 1	65.003 ± 14.329	21.163 ± 7.513	0.018
	A 0.5	T+A 0.01	27.935 ± 33.123	105.895 ± 21.821	0.034

A 1	T	10.081 ± 2.085	88.465 ± 2.556	0.000
A 1	T+A 0.01	10.081 ± 2.085	105.895 ± 21.821	0.016
A 1	T+A 0.1	10.081 ± 2.085	78.367 ± 17.299	0.020
T	T+A 0.5	88.465 ± 2.556	22.512 ± 9.852	0.005
T	T+A 1	88.465 ± 2.556	21.163 ± 7.513	0.002
T+A 0.01	T+A 0.5	105.895 ± 21.821	22.512 ± 9.852	0.011
T+A 0.01	T+A 1	105.895 ± 21.821	21.163 ± 7.513	0.014
T+A 0.1	T+A 0.5	78.367 ± 17.299	22.512 ± 9.852	0.015
T+A 0.1	T+A 1	78.367 ± 17.299	21.163 ± 7.513	0.017

Table S3 - Pairwise comparisons of the EGFR and HER2 activation in NCI-N87 cells between different stimulation times. Cells were treated with 5 µg/ml trastuzumab and/or 0.5 µM afatinib for different stimulation times. Western blot analyses were done in biological replicates. Two-sided Welch t-test and one-sample t-test were used. Significant p-values are shown, relevant comparisons are highlighted in bold. Gray and marked (*) are samples with only two biological replicates. Abbreviations: Ctrl: Control (untreated); T: 5 µg/ml trastuzumab; A: 0.5 µM afatinib; min: minutes

Activation of protein	Groups (X vs. Y)		Mean ± SD (X)	Mean ± SD (Y)	p-value
	X	Y			
EGFR	Ctrl	A 5 min	100	34.544 ± 4.662	0.002
	Ctrl	A 20 min	100	6.338 ± 7.811	0.002
	Ctrl	A 60 min	100	4.530 ± 6.146	0.001
	Ctrl	A 120 min	100	3.858 ± 3.676	0.000
	Ctrl	T+A 20 min	100	13.841 ± 15.348	0.010
	Ctrl	T+A 60 min	100	4.619 ± 5.429	0.001
	Ctrl	T+A 120 min	100	9.028 ± 9.739	0.004
	T 5 min	A 20 min	114.987 ± 41.273	6.338 ± 7.811	0.041
	T 5 min	A 60 min	114.987 ± 41.273	4.530 ± 6.146	0.041
	T 5 min	A 120 min	114.987 ± 41.273	3.858 ± 3.676	0.042
	T 5 min	T+A 20 min	114.987 ± 41.273	13.841 ± 15.348	0.038
	T 5 min	T+A 60 min	114.987 ± 41.273	4.619 ± 5.429	0.042
	T 5 min	T+A 120 min	114.987 ± 41.273	9.028 ± 9.739	0.041
	T 60 min	A 20 min	84.089 ± 22.515	6.338 ± 7.811	0.018
	T 60 min	A 60 min	84.089 ± 22.515	4.530 ± 6.146	0.020
	T 60 min	A 120 min	84.089 ± 22.515	3.858 ± 3.676	0.023
	T 60 min	T+A 20 min	84.089 ± 22.515	13.841 ± 15.348	0.015
	T 60 min	T+A 60 min	84.089 ± 22.515	4.619 ± 5.429	0.021
	T 60 min	T+A 120 min	84.089 ± 22.515	9.028 ± 9.739	0.017
	T 120 min	A 20 min	61.254 ± 16.818	6.338 ± 7.811	0.017
	T 120 min	A 60 min	61.254 ± 16.818	4.530 ± 6.146	0.018
	T 120 min	A 120 min	61.254 ± 16.818	3.858 ± 3.676	0.023
	T 120 min	T+A 20 min	61.254 ± 16.818	13.841 ± 15.348	0.023
	T 120 min	T+A 60 min	61.254 ± 16.818	4.619 ± 5.429	0.020

	T 120 min	T+A 120 min	61.254 ± 16.818	9.028 ± 9.739	0.016
	A 5 min	A 20 min	34.544 ± 4.662	6.338 ± 7.811	0.010
	A 5 min	A 60 min	34.544 ± 4.662	4.530 ± 6.146	0.003
	A 5 min	A 120 min	34.544 ± 4.662	3.858 ± 3.676	0.001
	A 5 min	T+A 60 min	34.544 ± 4.662	4.619 ± 5.429	0.002
	A 5 min	T+A 120 min	34.544 ± 4.662	9.028 ± 9.739	0.029
HER2	Ctrl	A 20 min	100	16.572 ± 17.240	0.014
	Ctrl	A 60 min	100	9.840 ± 9.892	0.004
	Ctrl	A 120 min	100	10.874 ± 9.548	0.004
	Ctrl	T+A 20 min	100	18.713 ± 15.015	0.011
	Ctrl	T+A 60 min*	100	6.415 ± 4.623	0.022
	Ctrl	T+A 120 min*	100	8.772 ± 7.458	0.037
	T 5 min	A 20 min	93.466 ± 20.930	16.572 ± 17.240	0.009
	T 5 min	A 60 min	93.466 ± 20.930	9.840 ± 9.892	0.010
	T 5 min	A 120 min	93.466 ± 20.930	10.874 ± 9.548	0.010
	T 5 min	T+A 20 min	93.466 ± 20.930	18.713 ± 15.015	0.009
	T 5 min	T+A 60 min*	93.466 ± 20.930	6.415 ± 4.623	0.014
	T 5 min	T+A 120 min*	93.466 ± 20.930	8.772 ± 7.458	0.011

Table S4 - Alterations in the cell lines NCI-N87, MKN1 and MKN7. Mutations and types of mutation are listed as well as CNV, Expression level (Z-Score) and Average Ploidy. Data are selected from COSMIC Cell Line Project, canSAR^a database and Arienti et al.^b (Forbes et al. 2015, Tym et al. 2016, Arienti et al. 2016). Abbreviations: N/A: not available

Cell line	Mutation Gene	AA mutation	Type	CNV Gene	Expression level (Z-Score)	Copy number	Average ploidy
NCI-N87	<i>ERBB2</i>	p.F425L ^a	Substitution-Missense	<i>ERBB2</i>	3.62	14	1.81
		p.S413L ^a	Substitution-Missense				
		p.L436V ^a	Substitution-Missense				
		p.D417D ^a	Substitution-Coding silent				
		p.T900P ^b	Substitution-Missense				
		p.R898G ^b	Substitution-Missense				
		p.Q902K ^b	Substitution-Missense				
		p.R897G ^b	Substitution-Missense				
		p.Q902R ^b	Substitution-Missense				
	<i>CHEK2</i>	p.Q330Q	Substitution-Coding silent	<i>GSK3A</i>	2.35	2 ^a	1.81
<i>PDGFRB</i>	p.P721P	Substitution-Coding silent	<i>MAPK14</i>	-3.05	1 ^a	1.81	
	<i>TP53</i>	p.R248Q	Substitution-Missense	<i>MTOR</i>	-2.69	N/A	1.81
		p.R155Q	Substitution-Missense				
	MKN1	<i>PIK3CA</i>	p.E545K ^a	Substitution-Missense	<i>ERBB2</i> ^a	N/A	3
p.V143A			Substitution-Missense	<i>PRKAA1</i>	4.34	5 ^a	2.98
<i>TP53</i>		p.R210H	Substitution-Missense				
MKN7	<i>EGFR</i>	p.I923I	Substitution-Coding silent	<i>ERBB2</i>	3.19	14	3.02
		p.L858R	Substitution-Missense	<i>JUN</i>	2.23	4 ^a	3.02
				<i>PRKAA1</i>	2.58	6 ^a	3.02
	<i>TP53</i>	p.278S	Substitution-Missense	<i>YES1</i>	2.12	5 ^a	3.02

Arienti, C., M. Zanoni, S. Pignatta, A. Del Rio, S. Carloni, M. Tebaldi, G. Tedaldi, and A. Tesei. 2016. "Preclinical evidence of multiple mechanisms underlying trastuzumab resistance in gastric cancer." *Oncotarget* 7 (14):18424-39. doi: 10.18632/oncotarget.7575.

Forbes, S. A., D. Beare, P. Gunasekaran, K. Leung, N. Bindal, H. Boutselakis, M. Ding, S. Bamford, C. Cole, S. Ward, C. Y. Kok, M. Jia, T. De, J. W. Teague, M. R. Stratton, U. McDermott, and P. J.

- Campbell. 2015. "COSMIC: exploring the world's knowledge of somatic mutations in human cancer." *Nucleic Acids Res* 43 (Database issue):D805-11. doi: 10.1093/nar/gku1075.
- Tym, J. E., C. Mitsopoulos, E. A. Coker, P. Razaz, A. C. Schierz, A. A. Antolin, and B. Al-Lazikani. 2016. "canSAR: an updated cancer research and drug discovery knowledgebase." *Nucleic Acids Res* 44 (D1):D938-43. doi: 10.1093/nar/gkv1030.

Table S5 - Pairwise comparisons of metabolic activity between trastuzumab (T), afatinib (A) and trastuzumab + afatinib (T+A) treated NCI-N87, MKN1, MKN7 and Hs746T cells. Cells were treated with trastuzumab, afatinib, trastuzumab + afatinib, trastuzumab solvent, afatinib solvent or trastuzumab + afatinib solvent. Two-sided Welch t-test and one-sample t-test were used. Significant p-values are shown.

Cell line	Groups (X vs. Y)		Mean ± SD (X)	Mean ± SD (Y)	p-value
	X	Y			
NCI-N87	Ctrl	A	100	35.416 ± 3.966	0.001
	Ctrl	T+A	100	36.231 ± 4.530	0.002
	T	A	92.041 ± 7.763	35.416 ± 3.966	0.002
	T	T+A	92.041 ± 7.763	36.231 ± 4.530	0.001
	Solvent T	A	98.007 ± 11.888	35.416 ± 3.966	0.007
	Solvent T	T+A	98.007 ± 11.888	36.231 ± 4.530	0.006
	A	Solvent A	35.416 ± 3.966	99.941 ± 11.609	0.006
	A	Solvent T+A	35.416 ± 3.966	97.395 ± 15.688	0.016
	Solvent A	T+A	99.941 ± 11.609	36.231 ± 4.530	0.005
	T+A	Solvent T+A	36.231 ± 4.530	97.395 ± 15.688	0.015
MKN1	Ctrl	A	100	67.078 ± 6.441	0.013
	Ctrl	T+A	100	68.417 ± 2.066	0.001
	T	A	98.765 ± 3.859	67.078 ± 6.441	0.004
	T	T+A	98.765 ± 3.85	68.417 ± 2.066	0.001
	Solvent T	A	102.104 ± 5.981	67.078 ± 6.441	0.002
	Solvent T	T+A	102.104 ± 5.981	68.417 ± 2.066	0.006
	A	Solvent A	67.078 ± 6.441	106.387 ± 7.298	0.002
	A	Solvent T+A	67.078 ± 6.441	103.631 ± 7.403	0.003
	Solvent A	T+A	106.387 ± 7.298	68.417 ± 2.066	0.008
	T+A	Solvent T+A	68.417 ± 2.066	103.631 ± 7.403	0.010
MKN7	Ctrl	T	100	106.189 ± 1.335	0.015
	Ctrl	Solvent T	100	107.701 ± 0.979	0.005
	T	A	106.189 ± 1.335	85.796 ± 8.316	0.048
	Solvent T	A	107.701 ± 0.979	85.796 ± 8.316	0.043
	A	Solvent A	85.796 ± 8.316	108.610 ± 6.179	0.022
	A	Solvent T+A	85.796 ± 8.316	108.311 ± 3.612	0.028

Solvent A	T+A	108.610 ± 6.179	88.076 ± 8.887	0.036
T+A	Solvent T+A	88.076 ± 8.887	108.311 ± 3.612	0.044

Table S6 - Pairwise comparisons of EGFR, MAPK and AKT activation between treatment (afatinib) and control (DMSO) in Hs746T and NCI-N87 cells. Cells were treated with afatinib or corresponding amount of DMSO as control. Two-sided Welch t-test and one-sample t-test were used. Significant p-values are shown. Abbreviations: Ctrl: Control (untreated); A: 0.5 μ M afatinib, DMSO: 0.05% DMSO

Cell line	Activation of protein	Groups (X vs. Y)		Mean \pm SD (X)	Mean \pm SD (Y)	p-value	
		X	Y				
Hs746T	MAPK	Ctrl	DMSO	100	71.251 \pm 7.546	0.022	
		A	DMSO	92.809 \pm 8.584	71.251 \pm 7.546	0.032	
	AKT	Ctrl	A	100	104.468 \pm 1.476	0.034	
NCI-N87	EGFR	Ctrl	A	100	5.280 \pm 2.503	< 0.001	
			DMSO	100	49.697 \pm 11.368	0.017	
		A	DMSO	5.280 \pm 2.503	49.697 \pm 11.368	0.017	
	MAPK	Ctrl	A	100	10.599 \pm 5.601	0.001	
			DMSO	100	81.846 \pm 6.942	0.045	
	AKT	Ctrl	A	DMSO	10.599 \pm 5.601	81.846 \pm 6.942	< 0.001
			A	DMSO	10.937 \pm 1.516	128.605 \pm 29.990	0.021