Supplementary Table S1

A

RD: CI for BI 2536 + VCR:

VCR \ BI 2536	2 nM	3 nM	4 nM	5 nM
1 nM	1.244	0.771	0.547	0.590
2 nM	0.668	0.510	0.483	0.660
3 nM	0.657	0.562	0.533	0.729

TE381.T : CI for BI 2536 + VCR:

VCR \ BI 2536	5 nM	6 nM	7 nM	8 nM
1 nM	0.722	0.403	0.389	0.352
2 nM	0.526	0.456	0.468	0.448
3 nM	0.538	0.590	0.599	0.599

A204: CI for BI 2536 + VCR:

VCR \ BI 2536	5 nM	6 nM	7 nM	8 nM
1 nM	1.437	1.191	1.023	0.950
2 nM	0.800	0.794	0.731	0.715
3 nM	0.540	0.525	0.567	0.616

RH30: CI for BI 2536 + VCR:

VCR \ BI 2536	5 nM	6 nM	7 nM	8 nM
1 nM	1.079	0.938	0.805	0.660
2 nM	0.795	0.685	0.543	0.539
3 nM	0.667	0.559	0.498	0.511

B

RD: CI for BI 2536 + VBL:

VBL \ BI 2536	2 nM	3 nM	4 nM	5 nM
0.5 nM	1.319	0.886	0.786	0.874
0.75 nM	1.198	0.669	0.664	0.853
1 nM	0.829	0.609	0.651	0.903

RD: CI for BI 2536 + VNR:

VNR \ BI 2536	2 nM	3 nM	4 nM	5 nM
1 nM	1.630	0.776	0.650	0.720
2 nM	1.205	0.509	0.583	0.687
3 nM	0.893	0.513	0.595	0.805

C

RD: CI for BI 6727 + VCR:

VCR \ BI 6727	6 nM	8 nM	10 nM
1 nM	1.204	1.083	0.960
2 nM	0.962	0.837	0.760
3 nM	0.869	0.801	0.720

TE381.T : CI for BI 6727 + VCR:

VCR \ BI 6727	5 nM	10 nM	15 nM
1 nM	1.095	0.729	0.656
2 nM	0.972	0.817	0.792
3 nM	0.991	0.861	0.858

D

RD-MCS: CI for BI 2536 + VCR:

VCR \ BI 2536	2 nM	3 nM	4 nM	5 nM
0.5 nM	0.985	1.393	1.297	1.003
1 nM	1.134	0.945	0.852	0.676
1.5 nM	0.816	0.604	0.495	0.369

E

Primary RMS: CI for BI 2536 + VCR:

VCR \ BI 2536	3 nM	5 nM	7 nM
1 nM	0.682	0.538	0.718
2 nM	0.478	0.478	0.772
3 nM	0.448	0.636	0.768

Supplementary Table S2

A RD: CI for BI 2536 + doxorubicin:

doxo \ BI 2536	2 nM	3 nM	4 nM	5 nM
0.1 µg/ml	1.085	1.306	0.948	1.459
0.2 µg/ml	1.999	2.138	2.296	2.170
0.3 µg/ml	0.863	0.935	0.920	0.919

TE381.T : CI for BI 2536 + doxorubicin:

doxo \ BI 2536	5 nM	6 nM	7 nM	8 nM
0.1 µg/ml	1.976	2.250	2.506	2.538
0.2 µg/ml	2.408	2.582	2.708	2.774
0.3 µg/ml	1.447	1.584	1.653	1.708

A204: CI for BI 2536 + doxorubicin:

doxo \ BI 2536	5 nM	6 nM	7 nM	8 nM
0.1 µg/ml	1.563	1.407	1.336	1.742
0.2 µg/ml	1.385	1.496	1.510	1.706
0.3 µg/ml	1.221	1.291	1.349	1.418

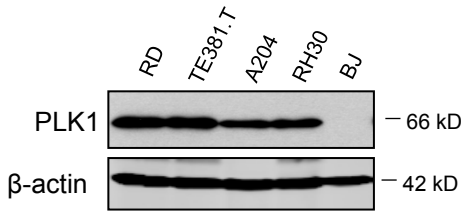
RH30: CI for BI 2536 + doxorubicin:

doxo \ BI 2536	5 nM	6 nM	7 nM	8 nM
0.1 µg/ml	1.513	1.308	1.463	1.834
0.2 µg/ml	2.094	1.449	1.369	2.652
0.3 µg/ml	1.111	1.176	1.411	1.543

B RD: CI for BI 2536 + taxol:

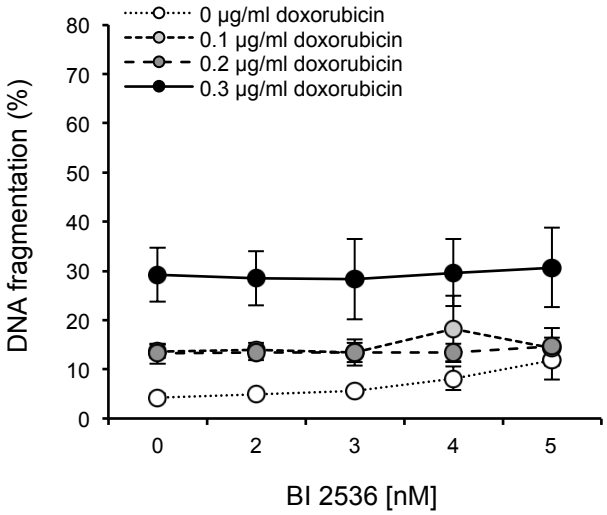
taxol \ BI 2536	2 nM	3 nM	4 nM	5 nM
2 nM	1.622	1.553	1.203	1.202
3 nM	1.225	1.213	0.966	0.989
4 nM	1.209	1.072	0.883	0.977

Supplementary Figure S1

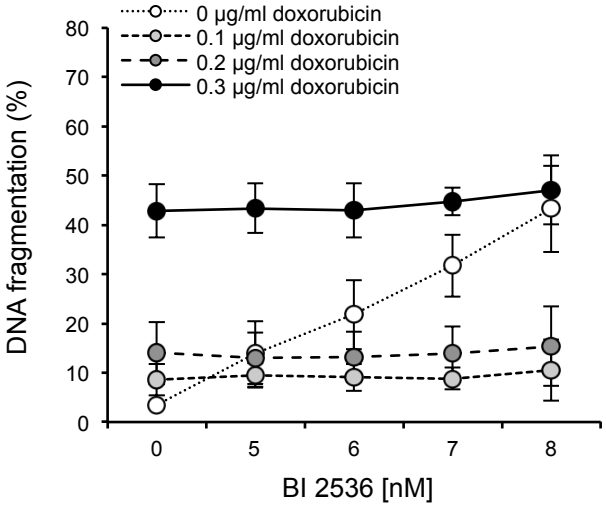


Supplementary Figure S2

A RD



TE381.T



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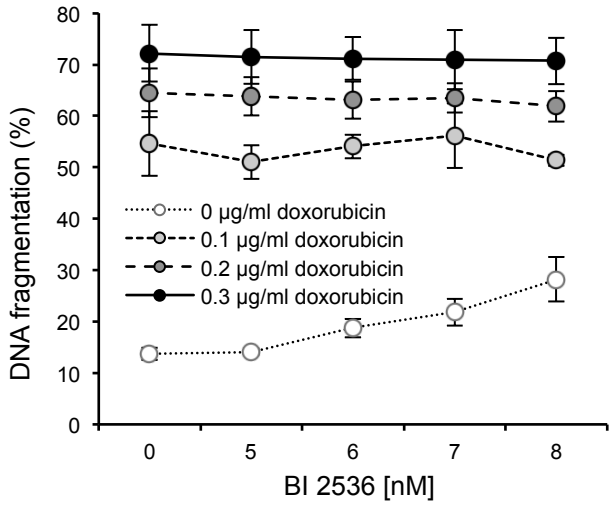
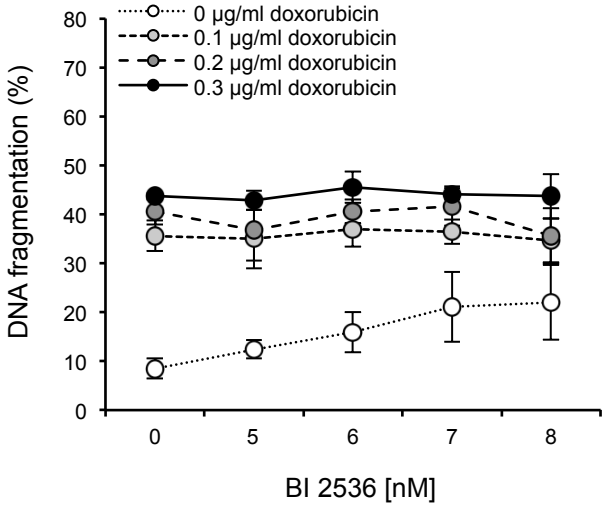
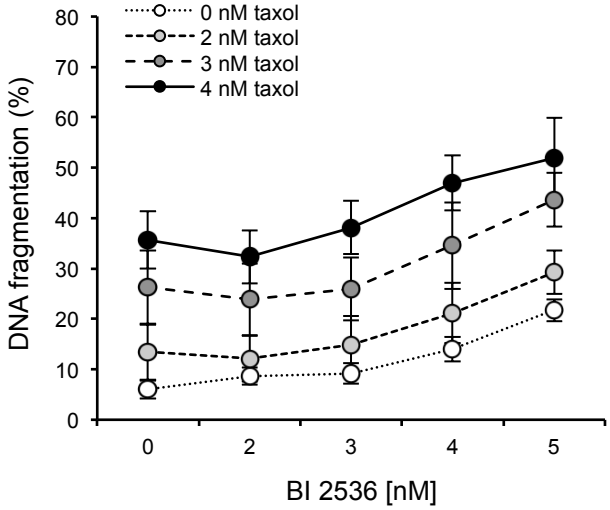


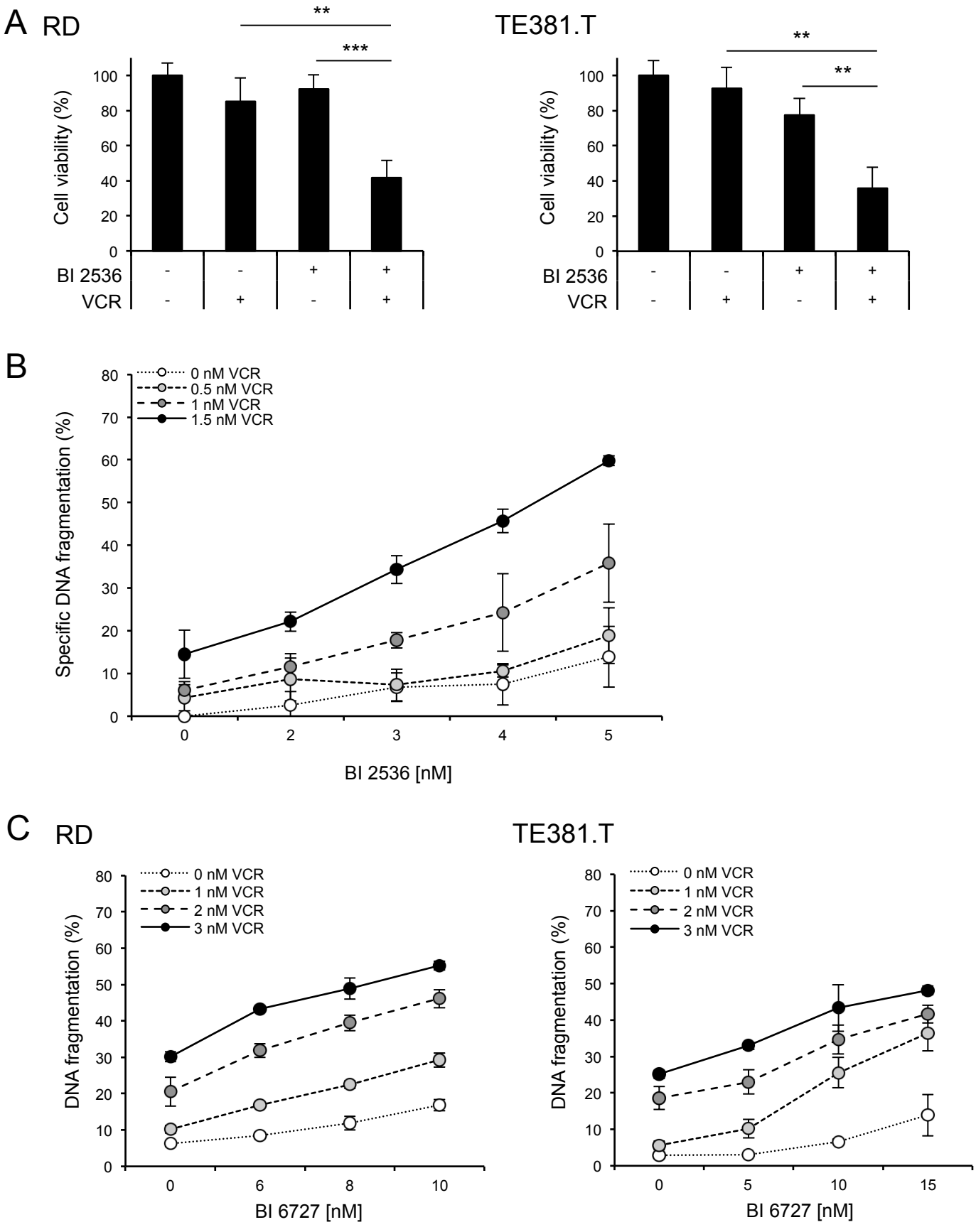
RH30



B RD

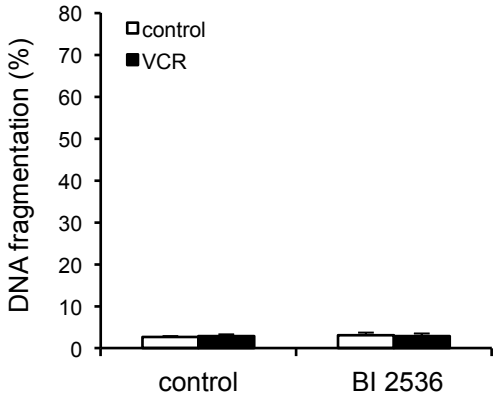


Supplementary Figure S3

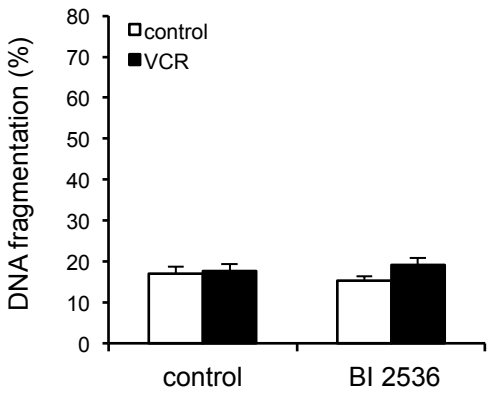


Supplementary Figure S3

D C2C12

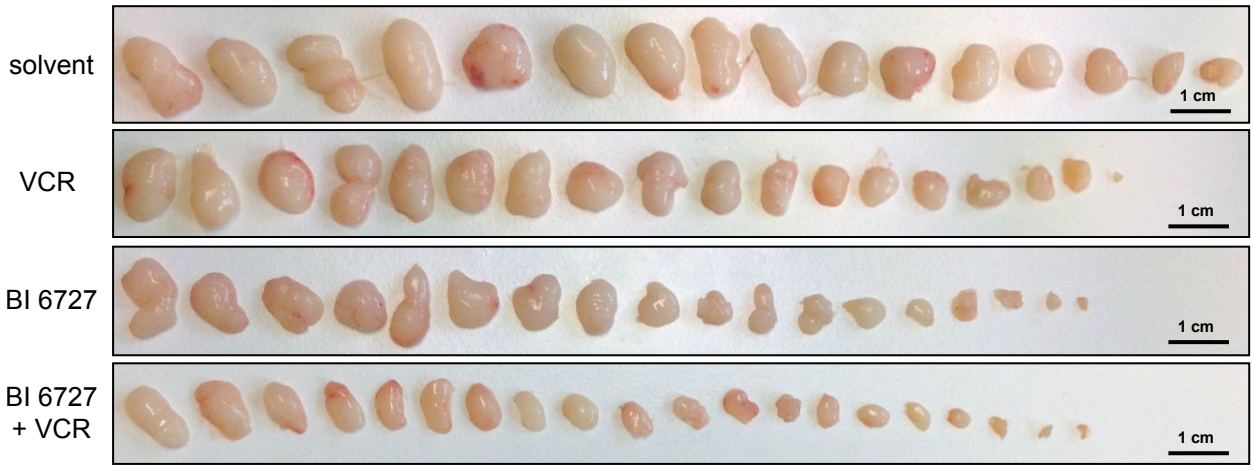


BJ



Supplementary Figure S4

A



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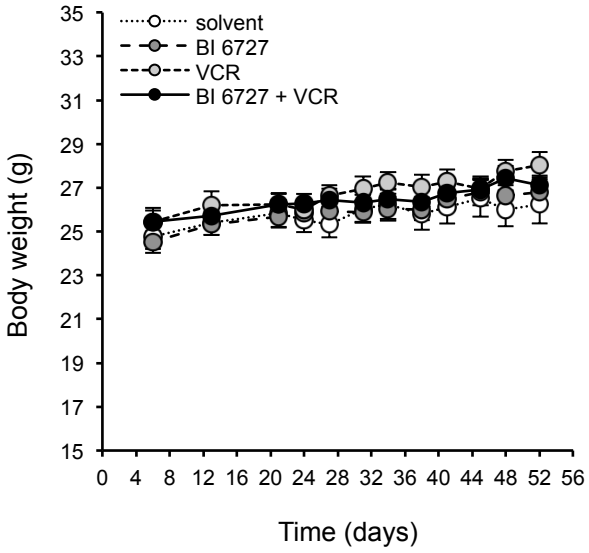
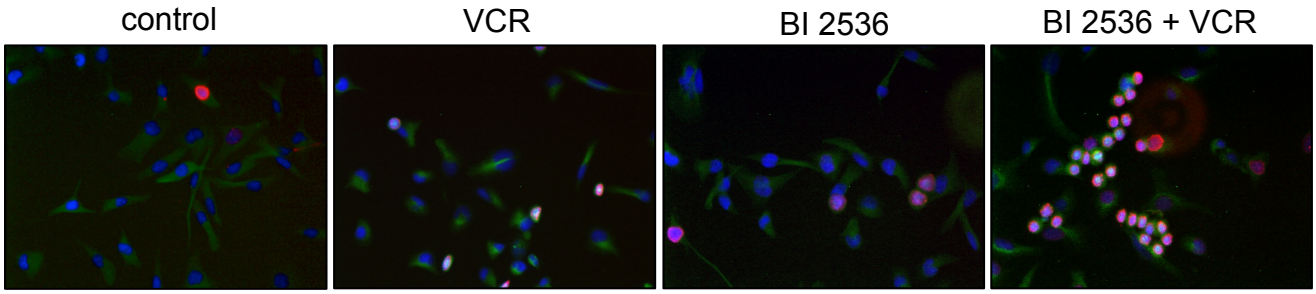
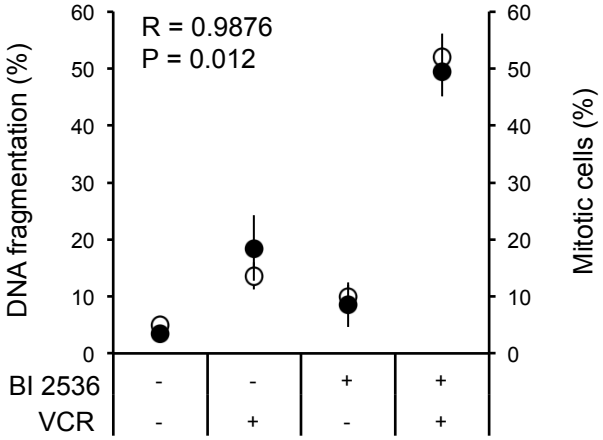


Supplementary Figure S5

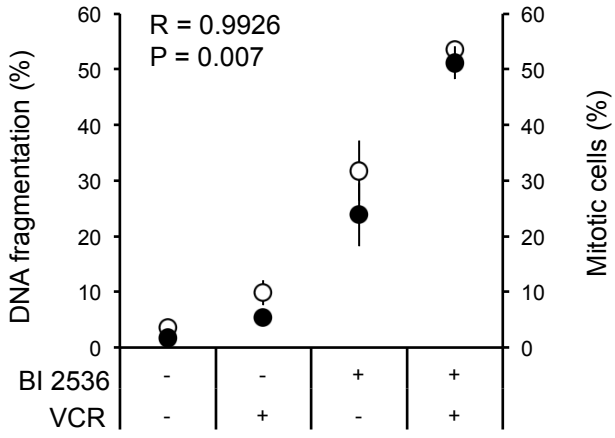
A RD



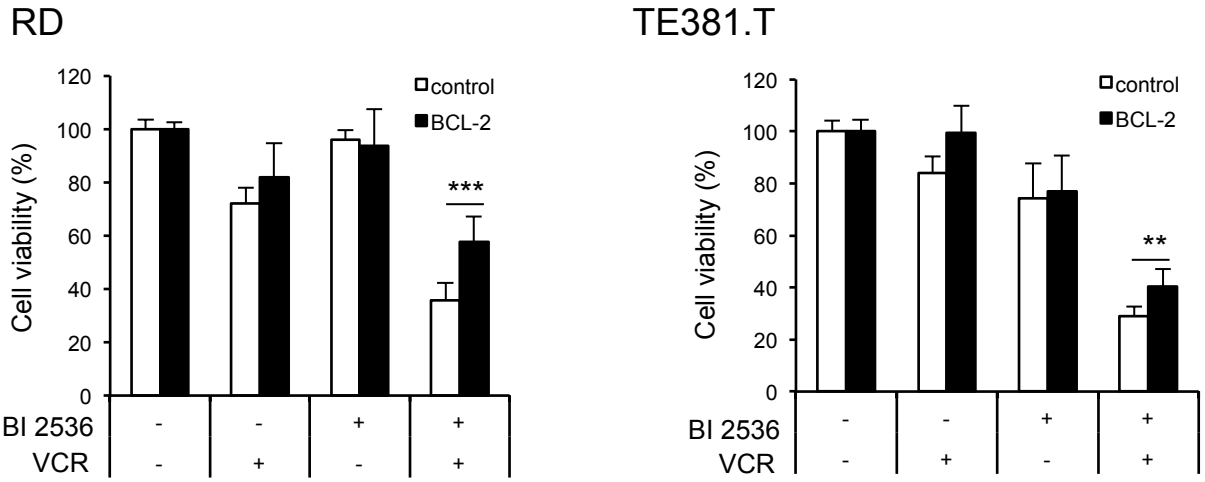
B RD



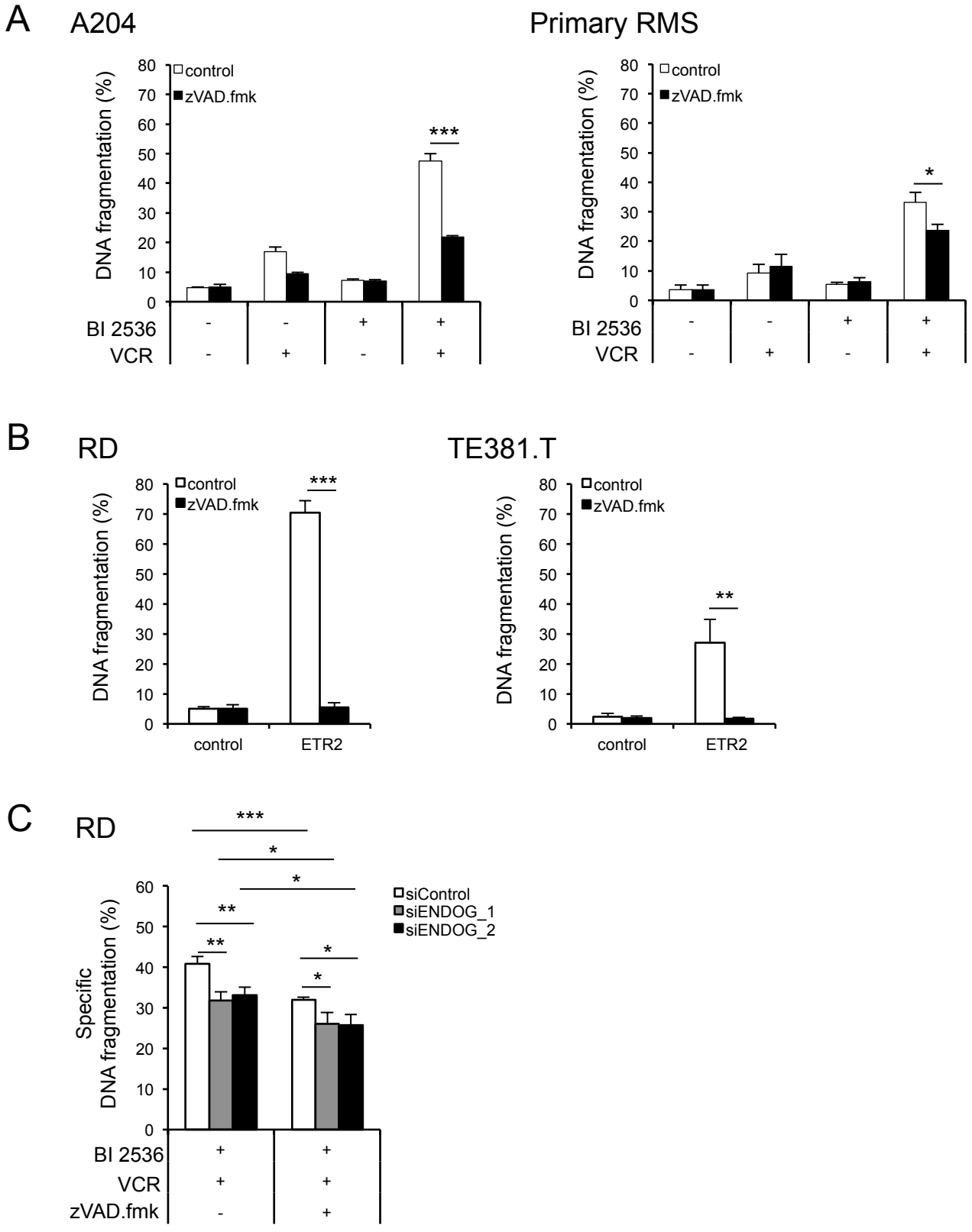
TE381.T



Supplementary Figure S6



Supplementary Figure S7



Supplementary Table Legends

Supplementary Table S1. CI values for individual drug combinations in different RMS cell lines. CI values for individual drug combinations of PLK1 inhibitors (BI 2536, BI 6727) and microtubule-destabilizing drugs (VCR, VBL, VNR) were determined by CalcuSyn software (Biosoft) for indicated concentrations. CI < 0.9 indicates synergism, 0.9 - 1.1 additivity and > 1.1 antagonism. **A**: List of CI values for apoptosis induction in RD, TE381.T, A204, and RH30 cells treated for 48 hours with different concentrations of BI 2536 and/or VCR. **B**: List of CI values for apoptosis induction in RD cells treated for 48 hours with different concentrations of BI 2536 and/or VBL or VNR. **C**: List of CI values for apoptosis induction in RD and TE381.T cells treated for 48 hours with different concentrations of BI 6727 and/or VCR. **D**: List of CI values for apoptosis induction in RD multi-cellular spheroid (MCS) cultures treated for 48 hours with different concentrations of BI 2536 and/or VCR. **E**: List of CI values for cell viability in primary ARMS cells treated for 48 hours with different concentrations of BI 2536 and/or VCR.

Supplementary Table S2. CI values for individual drug combinations in different RMS cell lines. CI values for individual drug combinations of PLK1 inhibitor BI 2536 and either doxorubicin (doxo) or taxol were determined by CalcuSyn software (Biosoft) for indicated concentrations. CI < 0.9 indicates synergism, 0.9 - 1.1 additivity and > 1.1 antagonism. **A**: List of CI values for apoptosis induction in RD, TE381.T, A204, and RH30 cells treated for 48 hours with different concentrations of BI 2536 and/or doxo. **B**: List of CI values for apoptosis induction in RD cells treated for 48 hours with different concentrations of BI 2536 and/or taxol.

Supplementary Figure Legends

Supplementary Figure S1. Analysis of PLK1 protein expression in RMS cell lines and non-malignant fibroblasts. Basal protein expression of PLK1 was determined by Western blotting in RD, TE381.T, A204, RH30 and BJ cells (n = 3). β-actin served as loading control.

Supplementary Figure S2. Evaluation of cytotoxicity of PLK1 inhibition in combination with chemotherapeutics in RMS. **A**: RD, TE381.T, A204 and RH30 cells were treated with indicated concentrations of the PLK1 inhibitor BI 2536 and/or doxorubicin (doxo). Apoptosis was determined at 48 hours by quantification of DNA fragmentation (n = 3). **B**: RD cells were treated with indicated concentrations of the PLK1 inhibitor BI 2536 and/or taxol. Apoptosis was determined at 48 hours by quantification of DNA fragmentation (n = 3). Results are expressed as mean + standard deviation (error bars).

Supplementary Figure S3. Evaluation of cytotoxicity of PLK1 inhibition in combination with microtubule-destabilizing drugs in RMS. **A**: RD and TE381.T cells were treated with indicated concentrations of the PLK1 inhibitor BI 6727 and/or VCR. Apoptosis was determined at 48 hours by quantification of DNA fragmentation (n = 3). **B**: RD and TE381.T cells were treated with 4 nM BI 2536 and/or 2 nM VCR and 7 nM BI 2536 and/or 1 nM VCR, respectively. Cell viability was analyzed at 48 hours using crystal violet assay (n = 3). **C**: RD cells were grown as multi-cellular spheroid (MCS) cultures and treated with indicated concentrations of BI 2536 and/or VCR. Apoptosis was determined at 48 hours by quantification of DNA fragmentation (n = 3). **D**: C2C12 myoblasts and BJ fibroblasts were treated with 7 nM BI 2536 and/or 2 nM VCR. Apoptosis was determined at 48 hours by quantification of DNA fragmentation (n = 3). Results are expressed as mean ± standard

deviation (error bars). Student *t* test was used to calculate two-sided *P* values. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Supplementary Figure S4. Effect of combined treatment with PLK1 inhibitors and VCR on non-malignant cells; and assessment of efficacy and toxicity *in vivo* in the human xenograft mouse model. **A**: Efficacy of BI 6727/VCR co-treatment *in vivo* in the human xenograft mouse model is displayed by images of the tumors dissected on the last day of the experiment. **B**: Body weight of the mice bearing human xenografts was monitored throughout treatment with BI 6727 and/or VCR as indicator of treatment toxicity. Results are expressed as mean ± standard deviation (error bars).

Supplementary Figure S5. Evaluation of mitotic arrest upon BI 2536/VCR co-treatment in rhabdomyosarcoma cell lines. Mitotic cells were determined at 18 hours using pH3, a specific marker of mitosis, upon treatment with BI 2536 and/or VCR in RD (treated with 4 nM BI 2536 and/or 2 nM VCR) and TE381.T (treated with 7 nM BI 2536 and/or 1 nM VCR) cells. **A**: Representative images of immunofluorescence staining of pH3 in RD cells are shown. **B**: Correlation coefficients and *P* values were calculated by regression analysis for DNA fragmentation upon 48 hours (n = 3) and mitotic arrest at 18 hours (n = 3) in RD and TE381.T cells.

Supplementary Figure S6. Analysis of involvement of pro- and antiapoptotic BCL-2 family proteins in BI 2536/VCR-induced cell death. RD cells were treated with 4 nM BI 2536 and/or 2 nM VCR, TE381.T cells with 7 nM BI 2536 and/or 1 nM VCR. Effects of BCL-2 overexpression on cell viability (n = 6 for RD; n = 5 for TE381.T) were analyzed by crystal violet assay at 48 hours of BI 2536/VCR co-treatment. Results are expressed as mean +

standard deviation (error bars). Student *t* test was used to calculate two-sided *P* values. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Supplementary Figure S7. Analysis of activation of caspase-dependent and caspase-independent cell death effector pathways in BI 2536/VCR-induced apoptosis. **A**: A204 and primary RMS cells were pretreated for one hour with 50 μM of broad-range caspase inhibitor zVAD.fmk and then treated with 6 nM BI 2536 and/or 3 nM VCR or 3 nM BI 2536 and/or 1 nM VCR, respectively. Apoptosis was analyzed at 48 hours by quantification of DNA fragmentation (n = 3). **B**: RD and TE381.T cells were pretreated for one hour with 20 μM of broad-range caspase inhibitor zVAD.fmk and then treated with 2 μg/ml or 4 μg/ml of TRAIL receptor-2 agonistic antibody ETR2, respectively. Apoptosis was analyzed at 48 hours by quantification of DNA fragmentation (n = 3). **C**: RD cells were transfected with siRNA against ENDOG (siENDOG) or non-silencing siRNA (siControl). Then, cells were pretreated for one hour with 20 μM of zVAD.fmk, co-treated for 48 hours with 4 nM BI 2536 and 2 nM VCR, and apoptosis was analyzed by quantification of DNA fragmentation (n = 3). Results are expressed as mean + standard deviation (error bars). Student *t* test was used to calculate two-sided *P* values. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.