

Supplementary Figures

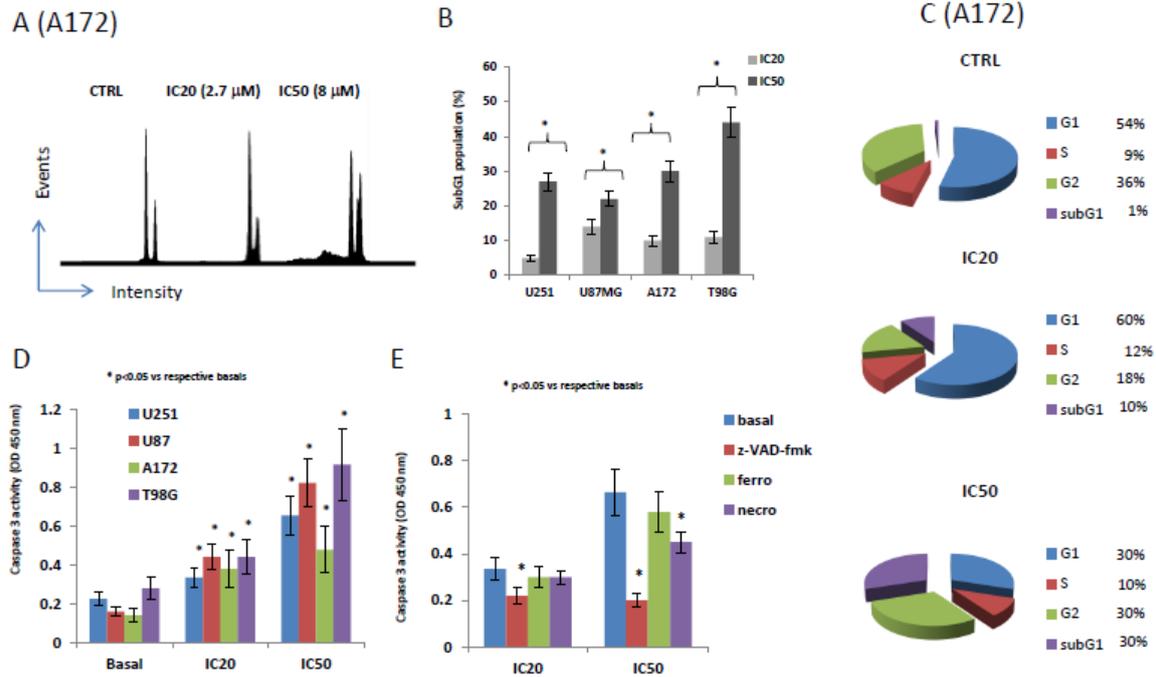


Figure S1. (A) Representative Propidium iodide staining performed in A172 cells determined at IC20 (2.7 μ M) and IC50 8.0 μ M); (B) SubG1 percentage in U87MG, U251, A172 and T98G: comparison between untreated cells and cells treated with RES529 at relative IC20 and IC50 values. (C) A further analysis revealed the accumulation in G1 cell cycle phase: in this panel is present the representative cell cycle distribution observed in A172 cells. (D) Caspase 3 activation after RES529 administration was increased in all treated cell lines. (E) Effects of z-VAD-fmk (pan-caspase inhibitor, 10 μ M), necrostatin-1 (necroptosis inhibitor, 50 μ M) and ferrostatin-1 (ferroptosis inhibitor, 5 μ M). For each assay, $n = 5$ (five replicate well/dishes). Data were presented as mean \pm standard deviation (SD). We use the symbol * to indicate the $p < 0.05$ for statistical comparisons.

U87MG subcutaneous xenografts

A Statistical analysis of Tumor weight

treatment	Tumor weight (mg) ± SD	statistics
CTRL	1091 ± 399	
25 mg/Kg	880 ± 302	P=0.1989 (NS)
50 mg/Kg	684 ± 231	P = 0.0120 vs CTRL P= 0.1204 (NS) vs 25 mg
100 mg/Kg	412 ± 110	P<0.0001 vs CTRL P= 0.0002 vs 25 mg P= 0.0035 vs 50 mg

B Statistical analysis of TTP

treatment	TTP (days) ± SD	statistics
CTRL	9.60 ± 2.46	
25 mg/Kg	9.00 ± 2.71	P=0.6103 (NS)
50 mg/Kg	13.40 ± 1.90	P < 0,001 vs CTRL. P= 0.005 vs 25 mg
100 mg/Kg	16.20 ± 2.39	P<0.0001 vs CTRL P<0.0001 vs 25 mg P= 0.0096 vs 50 mg.

C Hazard ratio analysis

comparison	Hazard ratio	CI 95%	LogRank test statistics
CTRL vs 25 mg/Kg	0.8735	0.36 to 2.10	P = 0.6909 (NS)
CTRL vs 50 mg/Kg	2.7320	1.02 to 7.35	P = 0.0030
CTRL vs 100 mg/Kg	3.9338	1.32 to 11.70	P < 0.0001
25 mg vs 50 mg/Kg	2.9228	1.067 to 7.99	P=0.0017
25 mg vs 100 mg/Kg	4.0545	1.35 to 12.18	P<0.0001
50 mg vs 100 mg/Kg	2.2365	0.91 to 6.24	P=0.0088

Figure S2. Statistical comparisons for charts showed in Figure 2. (A) tumor weight analyzed at the end of treatments with 25 mg, 50 mg and 100 mg/Kg/days. (B) statistical analysis for TTP data. (C) statistical analyses of hazard ratios.

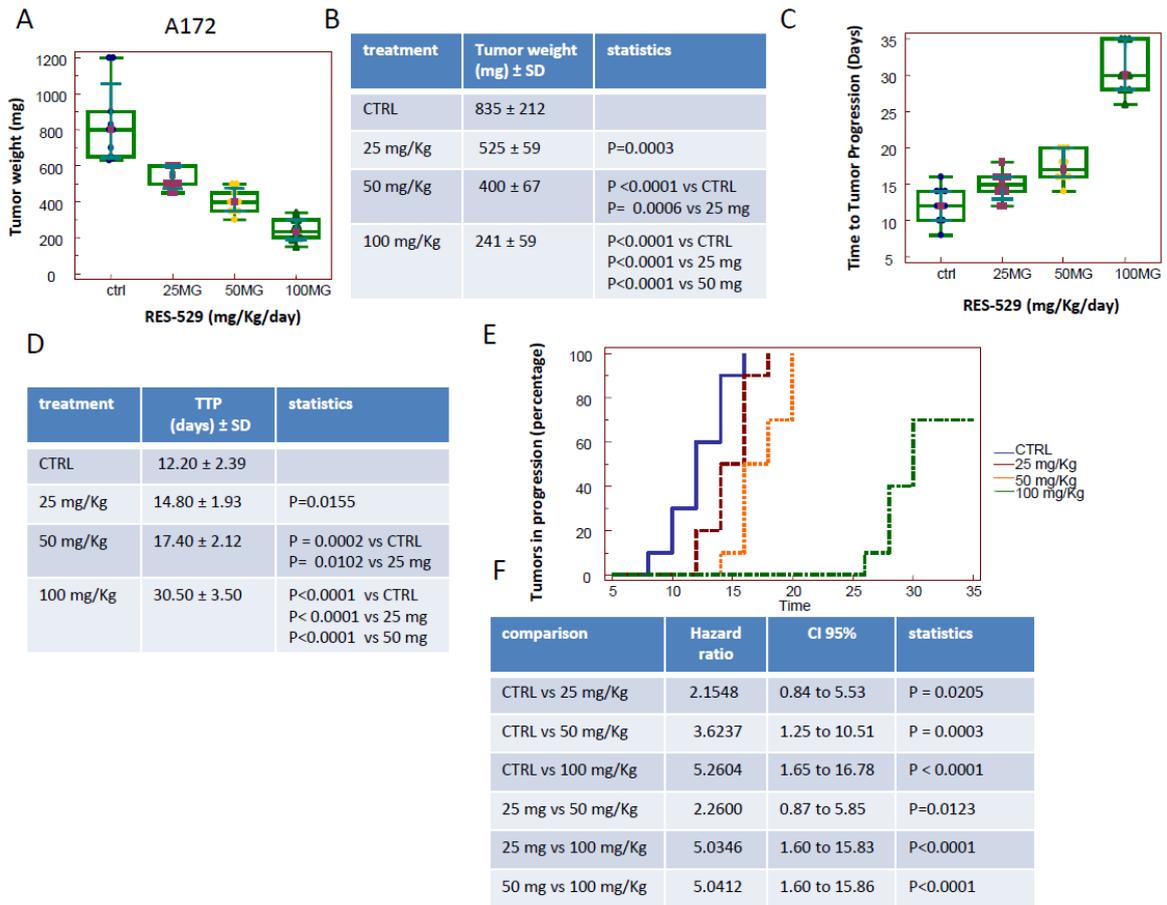


Figure S3. In Vivo experiments: RES529 inhibition of tumor growth of A172 cells subcutaneously injected in female nu/nu mice (xenograft model). 5 mice per group with two tumors each were considered (A) tumor weight analyzed at the end of treatments with 25 mg, 50 mg and 100 mg/Kg/days. (B) statistical analyses for tumor weight. (C) Time To Progression (TTP, days). (D) statistical analysis for TTP data. (E) Percentage of mice in progression plotted for time (Kaplan-Meier curves). (F) statistical analyses of hazard ratios.

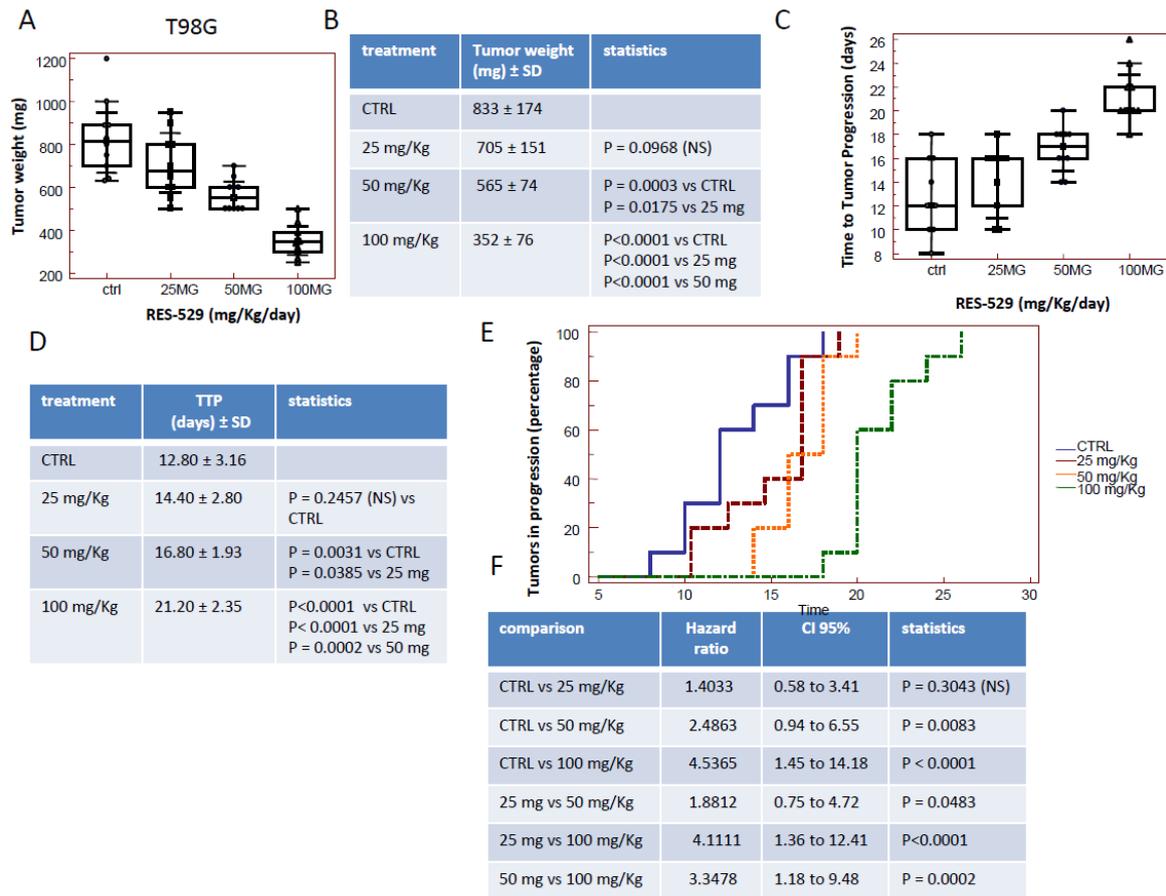


Figure S4 In Vivo experiments: RES529 inhibition of tumor growth of T98G cells subcutaneously injected in female nu/nu mice (xenograft model). 5 mice per group with two tumors each were considered. (A) tumor weight analyzed at the end of treatments with 25 mg, 50 mg and 100 mg/Kg/days. (B) statistical analyses for tumor weight. (C) Time To Progression (TTP, days). (D) statistical analysis for TTP data. (E) Percentage of mice in progression plotted for time (Kaplan-Meier curves). (F) statistical analyses of hazard ratios.

U87MG orthotopic intra-brain xenografts

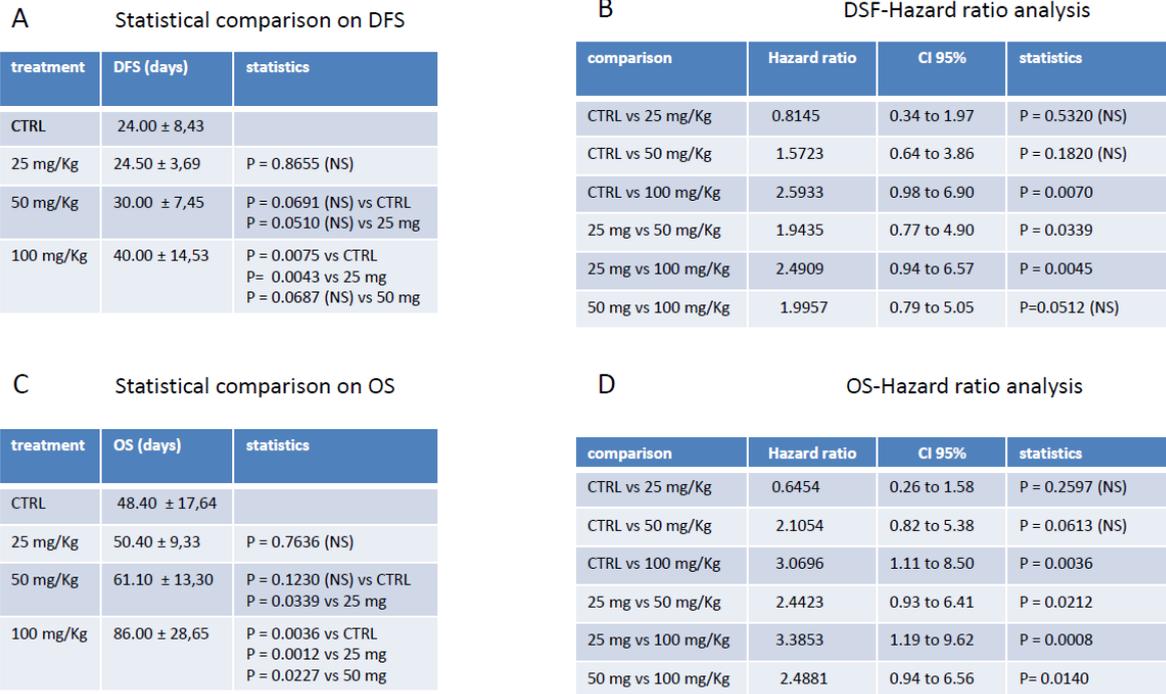


Figure S5. Statistical comparisons for charts showed in Figure 3. (A) Disease-free survival values. (B) statistical analyses of hazard ratios. (C) Overall Survival values and (D) calculation of Hazard ratios.

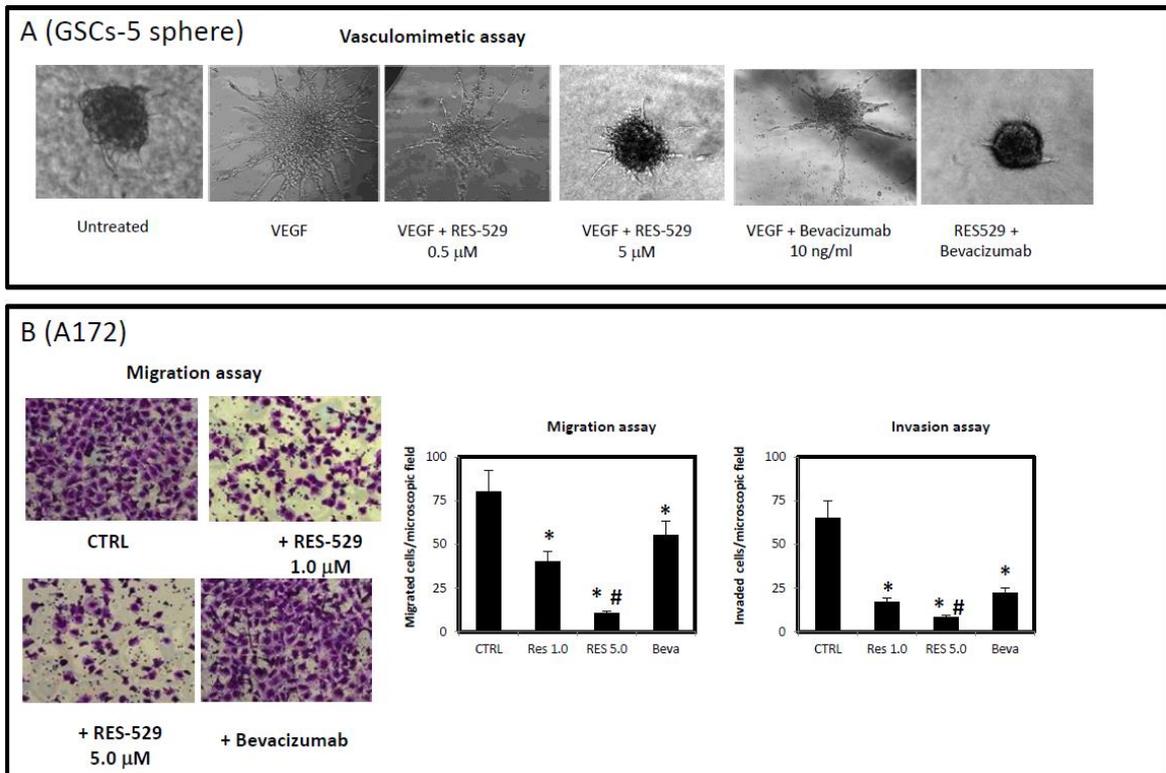


Figure S6. Anti-angiogenic effects of RES529. (A) vasculo-mimetic assay performed on patient derived Glioma stem like cell culture (GSCs-5) analyzed in presence of VEGF 10 ng/mL plus RES529 0.5 and 5.0 μM and/or bevacizumab 10 mg/mL. (B) migration and invasion assays evaluated in A172 cells and showing at the left exemplificative pictures of migrated cells and at right numeric evaluation of migrated and invaded cell per microscopic field. For each assay, $n = 5$ (five replicates). Data were

presented as mean \pm standard deviation (SD). Statistics: (B); * $p < 0.05$ vs CTRL and # $p < 0.05$ RES 5.0 μM vs. bevacizumab Experiments in this Figure were repeated three times, and each time the similar results were obtained.

A Statistical analysis of Tumor weight

treatment	Tumor weight (mg) \pm SD	statistics
CTRL	756 \pm 164	
RES-529	570 \pm 123	P=0.0101
Bevacizumab	480 \pm 150	P=0.0012 vs CTRL P = 0.0037 vs RES
RES + Beva	179 \pm 23	P<0.0001 vs CTRL P < 0.0001 vs RES P = 0.0002 vs Beva
Sunitinib	378 \pm 116	P<0.0001 vs CTRL P = 0.0021 vs RES
RES + Suni	160 \pm 72	P<0.0001 vs CTRL P < 0.0001 vs RES P<0.00001 vs Suni

B Statistical analysis of TTP

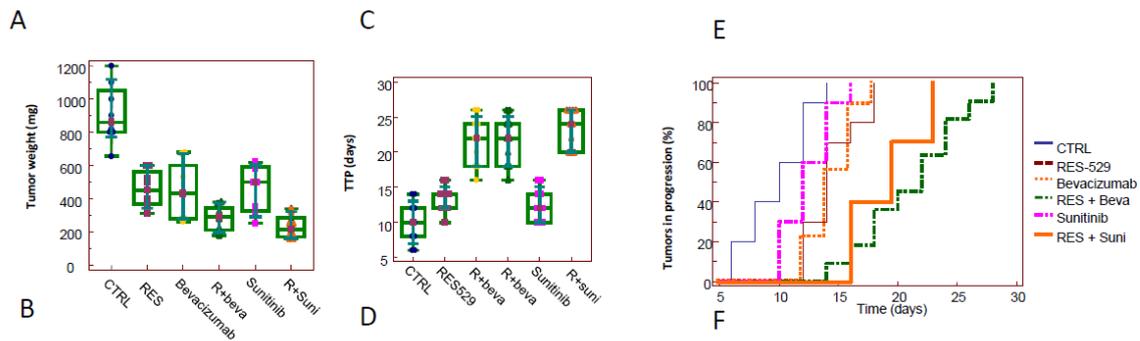
treatment	TTP (days) \pm SD	statistics
CTRL	12.20 \pm 2.39	
RES-529	15.40 \pm 2.12	P = 0.0054
Bevacizumab	14.90 \pm 0.88	P = 0.0036 vs CTRL P = 0.4992 (NS) vs RES
RES + Beva	22.60 \pm 3.53	P<0.0001 vs CTRL P<0.0001 vs RES P<0.0001 vs Beva
Sunitinib	15.60 \pm 3.24	P = 0.0156 vs CTRL P = 0.8720 (NS) vs RES P = 0.0575 (NS) vs Beva
RES + Suni	21.60 \pm 3.50	P < 0.0001 vs CTRL P < 0.0001 vs Suni P < 0.0001 vs RES P = 0.5331 (NS) vs Res + Beva

U87MG subcutaneous xenografts

C Hazard ratio analysis

comparison	Hazard ratio	CI 95%	statistics
CTRL vs RES-529	2.4781	0.97 to 6,85	P = 0.0050
CTRL vs Bevacizumab	2.0727	0.88 to 5,88	P = 0.0116
CTRL vs RES + Beva	4.8086	1.45 to 14,05	P < 0.0001
RES vs Beva	0.6080	0.25 to 1,50	P=0.1162 (NS)
RES vs RES + Beva	3.6119	1.25 to 10,46	P=0.0001
Beva vs RES + Beva	4.2393	1.40 to 12,93	P<0.0001
CTRL vs Sunitinib	2.2952	0.88 to 5,95	P = 0.0155
Suni vs RES	0.8062	0.33 to 1,94	P = 0.5222 (NS)
CTRL vs RES + Suni	4.5086	1.45 to 14,05	P<0.0001
Suni vs Suni + RES	3.1058	1.12 to 8,62	P=0.0010
RES vs RES + Suni	3.4412	1.20 to 9,83	P<0.0001
Suni + RES vs Beva + RES	1.1780	0.49 to 2,84	P = 0.5867(NS)

Figure S7. Statistical comparisons for charts showed in Figure 5. (A) tumor weight analyzed at the end of treatments with 50 mg mg/Kg/days RES-529 alone or without Bevacizumab or sunitinib as indicated in Figure 5A (B) statistical analysis for TTP data. (C) statistical analyses of hazard ratios.



treatment	Tumor weight (mg) ± SD	statistics
CTRL	909 ± 179	
RES-529	457 ± 113	P<0.0001
Bevacizumab	445 ± 170	P=0.0002 vs CTRL P = 0.8649 (NS) vs RES
RES + Beva	281 ± 76	P<0.0001 vs CTRL P = 0.0025 vs RES P = 0.0260 vs Beva
Sunitinib	462 ± 143	P=0.0002 vs CTRL P = 0.9394 (NS) vs RES
RES + Suni	230 ± 72	P<0.0001 vs CTRL P = 0.0011 vs RES P=0.00013 vs Suni

treatment	TTP (days) ± SD	statistics
CTRL	10.0 ± 2.98	
RES-529	12.90 ± 2.34	P = 0.0070
Bevacizumab	14.50 ± 1.58	P = 0.0006 vs CTRL P = 0.2163 (NS) vs RES
RES + Beva	21.60 ± 3.50	P<0.0001 vs CTRL P<0.0001 vs RES P<0.0001 vs Beva
Sunitinib	12.70 ± 3.02	P = 0.0431 vs CTRL P = 0.4096 (NS) vs RES P = 0.0575 (NS) vs Beva
RES + Suni	23.55 ± 3.67	P < 0.0001 vs CTRL P < 0.0001 vs Suni P < 0.0001 vs RES

comparison	Hazard ratio	CI 95%	statistics
CTRL vs RES-529	2.0917	1.09 to 8.31	P = 0.0011
CTRL vs Beva	3.0262	1.18 to 9.41	P = 0.0004
Beva vs RES	1.0105	0.42 to 2.43	P = 0.9734 (NS)
CTRL vs RES + Beva	5.3758	1.66 to 15.34	P < 0.001
RES + Beva vs RES	3.6127	1.25 to 10.47	P=0.0001
Beva vs RES + Beva	3.7386	1.28 to 10.94	P=0.0002
CTRL vs Sunitinib	1.9648	0.78 to 4.96	P = 0.0457
Suni vs RES	1.8506	0.74 to 4.63	P = 0.0626 (NS)
CTRL vs RES + Suni	4.3852	1.44 to 13.45	P < 0.0001
RES vs Suni + RES	3.3891	1.42 to 13.55	P<0.0001
Suni vs Suni + RES	3.9298	1.33 to 11.72	P<0.0001
Suni + RES vs Beva + RES	0.7771	0,32 to 1,88	P = 0,4683 (NS)

A172 cell model

Figure S8. In Vivo experiments: RES529 modified tumor growth of A172 cells subcutaneously injected in female nu/nu mice (xenograft model). Each group is represented by 5 mice with two tumors in the flank. Animals were tested with 50 mg mg/Kg/days RES-529 alone or without Bevacizumab or sunitinib as indicated in Figure 5A: (A) tumor weight analyzed at the end of treatments. (B) statistical analyses of tumor weight. (C) Time To Progression (TTP, days). (D) Statistical analyses on TTP. (E) Percentage of mice in progression plotted for time (Kaplan–Meyer curves). (F) statistical analyses of hazard ratios. (F) statistical analyses and HR determinations.

A Statistical comparison on DFS

treatment	DFS (days)	statistics
CTRL	50.40 ± 17.64	
RES-529	89.50 ± 24.20	P = 0.0006
Bevacirumab	79.20 ± 21.62	P = 0.0043 vs CTRL P = 0.3288 (NS) vs RES
RES + Beva	152.70 ± 54.28	P < 0.0001 vs CTRL P = 0.0035 vs RES P = 0.0009 vs Beva
Sunitinib	103.40 ± 29.19	P = 0.0002 vs CTRL P = 0.2615 (NS) vs RES
RES + Suni	162.60 ± 38.94	P < 0.0001 vs CTRL P = 0.0003 vs RES P = 0.0012 vs suni P = 0.6450 (NS) vs RES + Beva

B DSF-Hazard ratio analysis

comparison	Hazard ratio	CI 95%	LogRank test statistics
CTRL vs RES-529	3.2446	1.25 to 10.59	P = 0.0003
CTRL vs bevacizumab	3.0844	1.19 to 9.62	P = 0.0007
CTRL vs RES + Beva	6.8962	1.90 to 19.80	P < 0.0001
Beva vs RES	1.2347	0.51 to 2.98	P = 0.5730 (NS)
RES vs RES + Beva	4.2452	1.42 to 12.69	P = 0.0002
Beva vs RES + Beva	4.8299	1.58 to 15.39	P < 0.0001
CTRL vs Sunitinib	4.2044	1.49 to 14.90	P < 0.0001
Sunitinib vs RES	2.9514	1.08 to 8.09	P = 0.0039
CTRL vs RES + Suni	7.5674	2.51 to 21.59	P < 0.0001
RES vs RES + Sunitinib	5.2210	1.62 to 16.82	P < 0.0001
Suni vs RES + Suni	3.8890	1.32 to 11.41	P = 0.0009
RES + suni vs RES + beva	1.0938	0.39 to 3.03	P = 0.8592 (NS)

C Statistical comparison on Overall survival (OS)

Treatment	Overall survival (days)	statistics
CTRL	65.44 ± 18.54	
RES-529	123.25 ± 27.03	P < 0.0001
Bevacizumab	103.00 ± 24.01	P = 0.0011 vs CTRL P = 0.0942 (NS) vs RES
RES + Beva	200.70 ± 39.67	P < 0.0001 vs CTRL P = 0.0017 vs RES P = 0.0011 vs Beva
Sunitinib	174.82 ± 46.44	P < 0.0001 vs CTRL P = 0.0071 vs RES
RES + Suni	215.55 ± 33.33	P < 0.0001 vs CTRL P < 0.0001 vs RES P = 0.0371 vs suni P = 0.3783 (NS) vs RES + Beva

D DSF-Hazard ratio analysis

comparison	Hazard ratio	CI 95%	LogRank statistics
CTRL vs RES-529	4.2066	1.47 to 11.47	P < 0.0001
CTRL vs bevacizumab	3.3279	1.30 to 10.29	P = 0.0003
CTRL vs RES + Beva	7.7838	2.35 to 17.06	P < 0.0001
Beva vs RES	1.8054	0.72 to 4.51	P = 0.1504 (NS)
RES vs RES + Beva	5.2534	1.65 to 16.75	P < 0.0001
Beva vs RES + Beva	5.8393	1.77 to 19.31	P < 0.0001
CTRL vs Sunitinib	3.9396	1.53 to 15.93	P < 0.0001
Sunitinib vs RES	0.3355	0.122 to 0.92	P = 0.0042
CTRL vs RES + Suni	7.7838	2.35 to 22.06	P < 0.0001
RES vs RES + Sunitinib	5.9034	1.78 to 19.60	P < 0.0001
RES vs RES + Sunitinib	2.6424	0.98 to 7.15	P = 0.0293
RES + suni vs RES + beva	1.2275	0.3 to 3.52	P = 0.7002 (NS)

Figure S9. Statistical comparisons for charts showed in Figure 7 (U87MG). Animals were tested with 100 mg mg/Kg/days RES-529 alone or without Bevacizumab or sunitinib as indicated in Figure 7A. (A) Disease-free survival values. (B) statistical analyses of hazard ratios for DFS. (C) Overall Survival values and (D) calculation of Hazard ratios.

A Statistical comparison on DFS

treatment	DFS (days)	statistics
CTRL	27.00 ± 6.32	
RES-529	44.00 ± 21.06	P = 0.0250
Bevacizumab	32.50 ± 5.89	P = 0.0594 (NS) vs CTRL P = 0.1136 (NS) vs RES
RES + Beva	99.50 ± 80.53	P = 0.0109 vs CTRL P = 0.0493 vs RES P = 0.0172 vs Beva
Sunitinib	56.50 ± 12.48	P=0.0005 vsCTRL P = 0.1237 (NS) vs RES
RES + Suni	81.00 ± 33.15	P = 0.0006 vs CTRL P = 0.0080 vs RES P = 0.0422 vs suni P = 0.5103 (NS) vs RES + Beva

B DSF-Hazard ratio analysis

comparison	Hazard ratio	CI 95%	statistics
CTRL vs RES-529	2.2604	0.87 to 5.85	P = 0.0137
CTRL vs bevacizumab	1.8819	0.75 to 4.73	P = 0.0663(NS)
CTRL vs RES + Beva	4.8093	1.54 to 15.06	P<0.0001
Beva vs RES	1.1724	0.69 to 4.25	P = 0.1234 (NS)
RES vs RES + Beva	2.3746	0.90 to 6.28	P = 0.0267
Beva vs RES + Beva	4.7407	1.52 to 14.78	P<0.0001
CTRL vs Sunitinib	4.0709	1.35 to 12.25	P<0.0001
Sunitinib vs RES	0.7146	0.29 to 1.74	P = 0.3413
CTRL vs RES + Suni	4.3860	1.42 to 13.54	P<0.0001
RES vs RES + Sunitinib	2.2673	0.98 to 6.97	P = 0.0089
Suni vs RES + Suni	2.2389	0.90 to 6.15	P = 0.0180
RES + suni vs RES + beva	1.1726	0.46 to 2.96	P = 0.7125 (NS)

C Statistical comparison on Overall survival (OS)

treatment	OS (days)	statistics
CTRL	50.40 ± 17.64	
RES-529	89.50 ± 24.20	P = 0.0006
Bevacizumab	79.20 ± 21.62	P = 0.0043 vs CTRL P = 0.3288 (NS) vs RES
RES + Beva	152.70 ± 54.28	P<0.0001 vs CTRL P = 0.0035 vs RES P = 0.0009 vs Beva
Sunitinib	103.40 ± 29.19	P=0.0002 vs CTRL P = 0.2615 (NS) vs RES
RES + Suni	162.60 ± 38.94	P < 0.0001 vs CTRL P = 0.0003 vs RES P = 0.0012 vs suni P = 0.6450 (NS) vs RES + Beva

D DSF-Hazard ratio analysis

comparison	Hazard ratio	CI 95%	statistics
CTRL vs RES-529	3.0322	1.25 to 10.14	P = 0.0065
CTRL vs bevacizumab	2.6068	1.11 to 7.65	P = 0.0094
CTRL vs RES + Beva	5.8765	1.97 to 17.967	P<0.0001
Beva vs RES	1.7279	0.70 to 4.29	P = 0.1681 (NS)
RES vs RES + Beva	4.0153	1.36 to 11.89	P = 0.0003
Beva vs RES + Beva	5.0260	1.58 to 15.98	P<0.0001
CTRL vs Sunitinib	4.5556	1.46 to 14.25	P<0.0001
Sunitinib vs RES	0.7146	0.29 to 1.74	P = 0.3413
CTRL vs RES + Suni	4.9791	1.54 to 16.11	P<0.0001
RES vs RES + Sunitinib	4.8314	1.51 to 15.45	P<0.0001
Suni vs RES + Suni	3.5680	1.24 to 10.30	P = 0.0009
RES + suni vs RES + beva	0.9312	0.37 to 2.37	P = 0.8768 (NS)

Figure S10. Statistical comparisons for charts showed in Figure 8 (GSCs-5). Animals were tested with 100 mg mg/Kg/days RES-529 alone or without Bevacizumab or sunitinib as indicated in Figure 5A. (A) Disease-free survival values. (B) statistical analyses of hazard ratios for DFS. (C) Overall Survival and (D) calculation of Hazard ratios.