Supplementary Materials and Methods

Molecular Characterization of PDGFRA, KIT, *and* VEGFR2

DNA isolation was performed using TRIzol reagent (Invitrogen) as indicated by the manufacturer. The screening of mutations in the hotspot exons of *KIT* (exons 9, 11, 13, and 17) and *PDFGFRA* (exons 12, 14 and 18) was done by PCR–single-strand conformation polymorphism (SSCP), as previously described [1,2]. Assessment of *PDGFRA*, *KIT*, and *VEGFR2* gene amplification was performed by quantitative PCR, as previously described [1–3]. Primers and probes for *PDGFRA* and *KIT* amplification were previously described [1–3]. For VEGFR2, the primers and probes were the following: 5′-TGAAAGAGACACAGGAAATTACACTG-3′ (forward primer), 5′-CATAATAAATCTTGGGCAGAGAGG-3′ (reverse primer), 5′-CACAACCAGAGAGACCACATGGCTC-FL (donator probe), LC640-GCTTCTCCTTTGAAATGGGATTGGTAAGGA-3′ (acceptor probe).

Antibodies Used in the Western Blot and Array Validation

To assess the activation of intracellular signaling pathways, the antibodies used were the following: phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) (D13.14.4E); phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (D3F9); phospho-Akt (Ser^{473}) (D9E); phospho-Stat3 (Tyr^{705}) (D3A7); non–phospho-Src (Tyr^{527}); phospho-Src Family (Tyr^{416}). The membranes were stripped and incubated with antibodies to detect the total protein levels as follows: p44/42 MAP kinase (137F5); p38 MAP kinase; Akt (pan) (C67E7); Stat3; Src (36D10). An antibody against PARP expression was used for apoptosis detection. When necessary, β-actin (dilution 1:300; Santa Cruz Biotechnology, Dallas, TX) was used as a loading control.

For array validation, Western blot was performed with the same lysates used for the arrays to detect EGFR phosphorylation with the specific antibody phospho-EGFR (Y1068) (D7A5) from Cell Signaling Technology (Danvers, MA). The antibody to detect total EGFR (31G7; Zymed Laboratories, San Francisco, CA) was used as controls.

Supplementary References

[1] Gomes AL, Reis-Filho JS, Lopes JM, Martinho O, Lambros MB, Martins A, Schmitt F, Pardal F, and Reis RM (2007). Molecular alterations of KIT oncogene in gliomas. *Cell Oncol* **29**, 399–408.

[2] Martinho O, Longatto-Filho A, Lambros MB, Martins A, Pinheiro C, Silva A, Pardal F, Amorim J, Mackay A, Milanezi F, et al. (2009). Expression, mutation and copy number analysis of platelet-derived growth factor receptor A (PDGFRA) and its ligand PDGFA in gliomas. *Br J Cancer* **101**, 973–982.

[3] Martinho O, Goncalves A, Moreira MA, Ribeiro LF, Queiroz GS, Schmitt FC, Reis RM, and Longatto-Filho A (2008). KIT activation in uterine cervix adenosquamous carcinomas by KIT/SCF autocrine/paracrine stimulation loops. *Gynecol Oncol* **111**, 350–355.

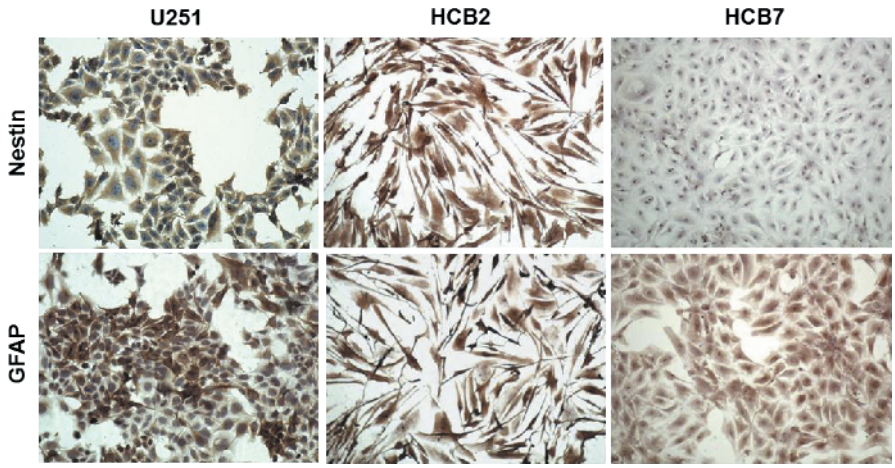

Figure W1. Immunocytochemistry analysis for Nestin and GFAP in primary cell lines (HCB2 and HCB7). U251 cell line was used as positive control. Immunocytochemistry was done with standard protocols, using specific primary antibodies for Nestin (1:200; Novus Biological, Cambridge, United Kingdom) and GFAP (1:1000; DakoCytomation, Glostrup, Denmark), followed by incubation with a biotinylated secondary antibody and revealed according to the streptavidin-biotin peroxidase complex system with visualization by 3,3′-diamonobenzidine chromogen. The pictures were taken at ×200 magnification.

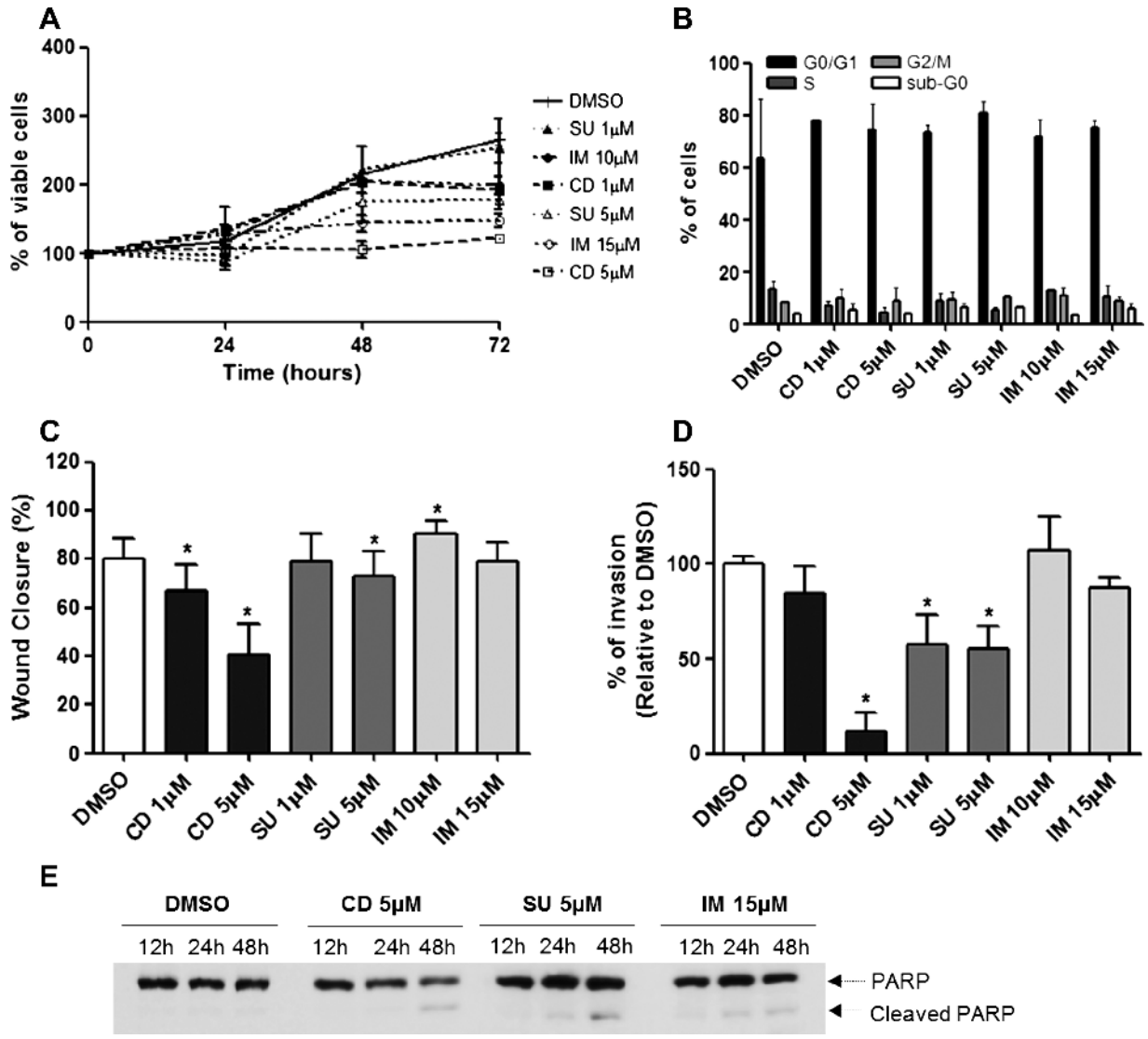

Figure W2. Effect of cediranib, sunitinib, and imatinib on SNB-19 cellular survival, cell cycle, migration, invasion, and apoptosis. (A) Cellular viability was measured at 24, 48, and 72 hours by MTS. (B) Cell cycle analysis was done at a 48-hour time point by flow cytometric analysis of PI-stained cells. (C) Migration was measured at 48 hours by wound healing migration assay. The relative migration distance was calculated as indicated in Materials and Methods section. (D) Invasion was assessed by matrigel invasion assay. (E) Apoptosis we assessed by Western blot for PARP cleavage. In all the experiments, the cells were incubated with one/two concentrations of each drug and also with the vehicle alone (DMSO) at the time points indicated. Data on A, B, C, and D are represented as the means ± SD, and differences with $P < .05$ on Student's t test were considered statistically significant (*).

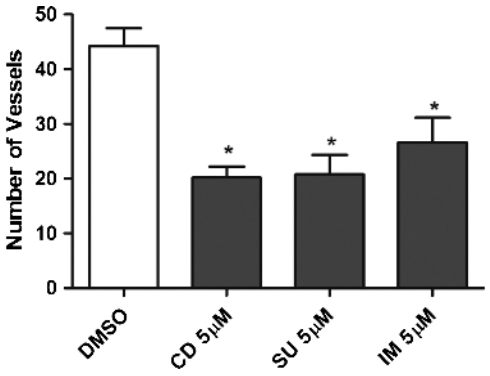

Figure W3. Effect of cediranib, sunitinib, and imatinib on angiogenesis. To assess angiogenesis, we performed CAM assay as described in Materials and Methods section. On day 14 of development, 20 μl of 0.5% FBS culture medium containing fixed concentration of the drugs (indicated in the graph) or DMSO alone was injected over the CAM. At day 17, the blood vessels were counted *ex ovo*. The results were expressed as the mean of the vessels counted for each group of treatments $\pm$ SD. Differences with $P <$ .05 on the Student's *t* test were considered statistically significant (*). A total of 20 eggs were used for angiogenesis assessment (five were treated with cediranib, five with sunitinib, five with imatinib, and five with DMSO alone).

Table W1. Molecular Alterations of RTKs in Glioblastoma Cell Lines.

Cell Line	Gene Amplification			Gene Mutations		RTK Phosphorylation*
	PDGFRA	*KIT*	*VEGFR2*	*PDGFRA*	*KIT*	
U251	Amp	Amp	Amp	wt	wt	EGFR; EphB2; PDGFRA; ROR1; EphA7; AXL
SNB-19	Amp	Amp	Amp	wt	wt	EGFR; PDGFRA; EphA7; AXL
U373	Amp	Amp	Amp	wt	wt	EGFR
SW1783	Amp	Amp	NA	wt	wt	EGFR; MSP; HER4; EphB2; PDGFRB; FGFR3; M-CSFR; ROR1
U-87 MG	NA	NA	NA	wt	wt	EGFR; MET; EphA7; MSP; HER4; EphB2; VEGFR2; FGFR3; M-CSFR; RET; ROR1
GAMG	Amp	NA	NA	wt	wt	EGFR; EphA7; FGFR3; AXL; KIT; MET; EphB2
SW1088	NA	NA	NA	wt	wt	EGFR; EphB2; PDGFRB; ROR1
A172	NA	NA	NA	wt	wt	EGFR; EphB2; PDGFRB; ROR1; AXL

Amp, amplified; NA, not amplified; wt, wild type.
*Assessed using a phospho_RTK array.

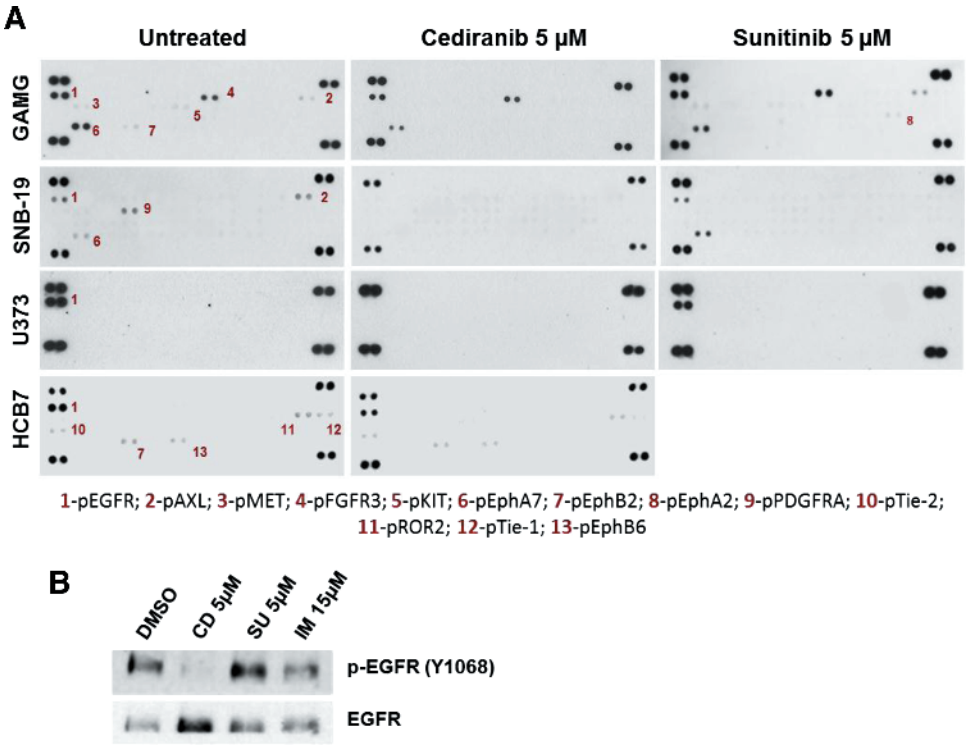

Figure W4. Cediranib and sunitinib targets in glioblastoma cells. (A) To identify the RTKs that are activated in the glioblastoma cell lines before and after cediranib and sunitinib treatment (5 μM by 2 hours), we used a phospho-RTK array (GAMG, SNB-19, U373, and HCB7 cell lines). Each RTK is represented in duplicate in the arrays (two spots side by side), and in addition, four pairs of phosphotyrosine positive controls are in the corners of each array. (B) To validate the arrays, we performed Western blot analysis with specific antibodies for EGFR phosphorylated proteins in U251 cell line treated with the three drugs.