Supplemental Figure 1. (A) DMS footprinting analyses (55) of the vegfr-2 promoter oligo: 5’ radiolabeled vegfr-2 promoter oligo was heat denatured and slowly cooled to room temperature in presence or absence of 100 mM KCl, allowing the guanine repeats to fold into G-quadruplexes. The resulting structures were treated with 0.5% DMS for 2 min to methylate the guanine residues in oligonucleotides. The methylated oligonucleotides were run and purified from native PAGE. AG represent chemical cleavage reaction specific to purine bases. The decrease of signal intensity in presence of KCl indicates that guanine residues are all protected by methylation. (B) CD spectra of mutated vegfr-2 promoter oligo (5’-GGG TAC CCG GGT GAG GGG CGG GG-3’) at 20 °C in absence (black line) and in presence of RHPS4 (gray line). (C) CD melting (filled circles) and annealing (filled triangles) profiles of vegfr-2 promoter sequence oligo recorded at 264 nm, and CD melting in presence of RHPS4 (empty circles). (D) Non-denaturing polyacrylamide gel electrophoresis (PAGE) of oligonucleotides in K+ containing solutions. a: [d(TGGGGT)]₄ as G-quadruplex marker; b: wt oligo; c: mut oligo. Nondenaturing gel electrophoresis was performed using 15% polyacrylamide gel (29:1 acrylamide:bisacrylamide ratio) containing 0.1 M Tris-Borate-EDTA (TBE) (Biorad) and 80 mM KCl, pH 7.0. Gel was run at 20 °C and 100 V for 2 h in 1xTBE running buffer supplemented with 80 mM KCl. A concentration of 50 μM was used for each sample. Glycerol was added (10% final) to facilitate sample loading in the wells. Bands in the gels were visualized by UV-shadowing.
Supplemental Figure 2. HUVEC cells treated with 0.5 µM of RHPS4 for the indicated times were collected and mRNAs expression was analyzed by RT-PCR. Histograms represent the percentage of inhibition in treated vs untreated samples, after cyclophilin normalization. Images show one representative of three independent experiments with similar results. Bars indicate means ±SD. Results showed that, among those analyzed, some G4 containig genes were down-regulated (vegf-A, vegfr-1, IL-1R, CXCR1 and AchRβ1) while others remained unchanged (EGFR and FGFR). As reported, PDGFRβ was not detectable in endothelial cells in that culture conditions (53). The analyzed non containing G4 genes were not modulated.
Supplemental Figure 3. (A) H5V cells, untreated (-) or treated with 0.5 µM RHPS4 for 72 hrs to achieve VEGFR-2 down-regulation, were starved for 24 hrs in presence or absence of RHPS4 and then processed for chemotaxis assays. Histograms represent the fold change of the number of migrated cells in VEGF-A stimulated vs unstimulated condition. (B) H5V cells treated as in A were processed for the in vitro angiogenesis assay. Representative images of tubular structures (TS) in the indicated samples are shown on the left panels (20X magnification). Quantification of TS are reported on the right. Histograms represent the mean number of branch points per field. Histograms show the mean values of three independent experiments while images show one representative of three independent experiments with similar results. Bars indicate means ±SD. *=p<0.1 **= p<0.01.
Supplemental Figure 4. (A) HUVEC cells untreated or treated with 0.5 μM for 72 hrs were processed for FACS analysis to evaluate cell cycle by PI staining and apoptosis by annexin V expression. (B) HUVEC cells treated with RHPS4 as above or with 0.5 μM camptothecins for 2 hrs were immunostained with the anti γH2AX antibody and counterstained with Hoechst for detection of DNA damage activation. Representative images at 100X magnification are shown. Histograms represent the percentage of γH2AX foci positive nuclei on total nuclei. (C) HUVEC cells untreated or treated as indicated for 72 hours were fixed with 2% formaldehyde and stained for β-galactosidase expression. Representative images at 20X magnification are shown. Histograms represent the percentage of β-galactosidase. Histograms in B (lower panel) and C (right panel) show the mean values of three independent experiments while histograms in A and images in B (upper panel) and C (right panels) show one representative of three independent experiments with similar results. Bars indicate means ±SD.
Supplemental Figure 5. (A) Polymerase stop assays: the oligonucleotide containing the G4-forming region of vegfr-2 (see material and methods) underwent polymerase reaction in presence of 5 mM KCl plus increasing concentrations of Emicoron (left panel) and PPL3C (right panel). The accumulation of stop products indicates the presence of stabilized G4 structures impeding the DNA-polymerase passage. (B) HUVEC cells untreated or treated with 0.5 μM Emicoron or PPL3C for 72 hrs were analysed for Immunofluorescence against VEGFR-2 and counterstained with Hoechst. Representative images at 63X magnification are shown. (C) HUVEC cells untreated (-) or treated as in B, were starved for 24 hrs in presence or absence of Emicoron or PPL3C and then processed for chemotaxis assays. Histograms represent the fold change of the number of migrated cells in VEGF-A stimulated vs unstimulated condition. (D) Matrigel, premixed with VEGF-A alone or in combination with 0.5 μM Emicoron or PPL3C, was injected subcutaneously into C57 BL/6 mice for the in vivo angiogenesis assay. Pictures show matrigel plugs removed 4 days post injection. Histograms represent the hemoglobin content measured in the relative samples expressed as absorbance (OD 540nm) per matrigel mgs. Histograms show the mean values of three independent experiments while images show one representative of three independent experiments with similar results. Bars indicate means ±SD.
Supplemental Figure 6. HUVEC cells infected with GFP, Rac1(L61) and RhoA(L63) carrying adenoviruses, were treated and processed for chemotaxis assay. Histograms represent the number of invading cells in treated samples expressed as percentage of untreated controls of each cell infected population. The experiment is average of three independent experiments performed in quadruplicate. Bars indicate means ±SD.