Supplemental Materials and Methods

Tumorigenicity in Nude Mice – Animals were housed in barrier facilities on a 12-hour light:dark cycle at the Dipartimento di Biologia e Patologia Cellulare e Mollecolare (University Federico II, Naples, Italy) and had free access to food and water. This study was conducted in accordance with Italian regulations for animal experimentation; manipulations were performed while animals were under isoflurane gas anesthesia. TT cells (1x10⁷/mouse) were inoculated subcutaneously into the right dorsal portion of 4-week-old male BALB/c nu/nu mice (Jackson Laboratories, Bar Harbor, ME). When established tumors developed, ZD6474 (50 mg/kg/day dissolved in PBS containing 0.5% v/v Tween 80) or vehicle was administered by oral gavage for five consecutive days/week. Tumor diameters were measured with calipers at regular intervals and tumor volumes (V) were calculated with the rotational ellipsoid formula: V=AxB²/2 (A=axial diameter; B= rotational diameter). Tumors were excised and divided in two parts. Half the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half was fixed overnight in neutral buffered formalin and processed for histological examination.

Supplemental results

Inhibition of TT-induced tumor formation in nude mice by ZD6474 – Nude mice were injected with TT (harbouring RET/C634W) cells and, when tumors measured ~200 mm³, animals (7 for each group) were randomly assigned to receive per os ZD6474 (50 mg/kg/day) or vehicle 5 days/week for 17 days. Tumor growth was monitored every 3–4 days with calipers. Treatment with ZD6474 greatly reduced tumor burden (Fig. 1). After 17 days of treatment, the mean size of tumors in mice treated with vehicle or ZD6474 was 560 mm³ [95% confidence interval (CI) = 406-715 mm³] and 26 mm³ (95% CI = 3-55 mm³), respectively (P < 0.0001). Next, we divided vehicle-treated animals into two groups: one continued to receive vehicle [n = 3] and the other group [n = 4] received ZD6474 for three weeks. ZD6474 exerted significant effects also on these large tumors (difference 460 mm³; P < 0.006). Tumor-growth inhibition was associated with a marked reduction of in vivo RET phosphorylation levels on tyrosine 1062 (Fig. 1, inset). Thus, in vivo, ZD6474 targeted the RET protein. By directly targeting VEGFRs in tumor endothelium, ZD6474 might also prevent the development of tumor neovascularization. Thus, blood vessels were counted upon staining with CD31 antibody; ZD6474 reduced the number of vessels (from 7±3.5 to 2±1 per microscopic field). Therefore, the anti-tumor effect of ZD6474 is associated with combinatory inhibition of both RET and angiogenesis.