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NOTE

RQN-18690A (18-deoxyherboxidiene) targets SF3b, a spliceosome component, and inhibits angiogenesis

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Angiogenesis is a complex process involving several distinct and sequential steps such as membrane degradation, migration (chemotaxis), proliferation and formation of capillary tubes in endothelial cells.[1,2] Abnormal angiogenesis often occurs in pathological conditions such as cancer, rheumatoid arthritis, diabetic retinopathy and other chronic inflammatory diseases. An important step in developing pathological angiogenesis involves producing vascular endothelial growth factor (VEGF) by normal and tumor cells and the subsequent hyperactivation of downstream signaling pathways in endothelial cells. In addition, VEGF-mediated signaling occurs in tumor cells expressing VEGF receptors, contributing to key aspects of tumorigenesis, including the function of cancer stem cells and tumor initiation.[3] Thus, inhibition of angiogenesis via VEGF-mediated pivotal signaling has been considered a promising strategy for the treatment of angiogenesis-related diseases including cancer. We previously discovered several new angiogenesis inhibitors, epoxyquinols A[4] and B,[5–9] epoxytwinol A,[10] azaspirene,[11,12] RK-805[13] and RK-95113[14] from the fungal metabolites, and performed a mode-of-action study. In our continuous screening for angiogenesis inhibitors from microbial origin, we found a potent angiogenesis inhibitor, RQN-18690A (18-deoxyherboxidiene), from the culture broth of *Streptomyces* sp. QN18690 (Figure 1). Herein we revealed that 18-deoxyherboxidiene inhibits the angiogenic process and targets SF3b, a spliceosome component that is a subcomplex of the U2 small nuclear ribonucleoprotein (snRNP) in the spliceosome.

We performed a high-throughput wound-healing screening using human umbilical vein endothelial cells (HUVECs) to identify an effective hit broth.[15] HUVECs (1×10^4 cells) in HuMedia-EG2 (Kurabo, Osaka, Japan) with 0.1% fetal bovine serum (FBS) were plated in a 96-well plate and incubated to confluence in 5% CO_2 at 37 °C. The cell monolayer was scratched using a pipette tip. After replacing the medium with fresh medium containing 3.1 ng ml^{-1} of VEGF (R&D Systems, Minneapolis, MN, USA) to remove cell debris, the test broth or vehicle was added to the cells and VEGF-induced cell motility was observed under a phase contrast microscope (Olympus, Tokyo, Japan). The results showed that the culture broth of *Streptomyces* sp. QN18690 strongly inhibited the wound-healing process. Ethyl acetate extract of the culture broth (2.5 l) was applied to a silica gel column chromatography and eluted with $CHCl_3$-MeOH (100:1, 50:1, 20:1, 10:1, 5:1 and 1:1 v/v). The eluents with $CHCl_3$-MeOH (50:1) were further purified by RP-HPLC (PEGASIL ODS (Senshu Scientific Co. Ltd., Tokyo, Japan), $\phi 20 \times 250$ mm, 40% aq to 100% MeCN in a linear gradient, 9.0 ml min^{-1}) to obtain RQN-18690 A (0.5 mg) as a colorless oil, $[\alpha]_D^{22} = +12.2$ ($c = 0.33$, MeOH), UV (MeOH)λmax (log ε) 236 (2.53) nm. The MW was established as $C_{25}H_{42}O_5$ with five degrees of unsaturation by high-resolution FAB-MS (m/z 421.2969 [M-H]$^-$, calculated for $C_{25}H_{41}O_5$, 421.2954). The IR spectrum (neat) showed characteristic absorptions at 3550, 2950, 1700, 1650, 1545, 1505, 1455, 1420 and 1100 cm^{-1}, suggesting the presence of carboxylic acid. The chemical structure of RQN-18690A was unambiguously determined as 18-deoxyherboxidiene by the physico-chemical properties and extensive NMR analysis (Supplementary Table S1).[16]

As 18-deoxyherboxidiene inhibited VEGF-induced motility in the wound-healing assay, we next examined its inhibitory effect in a HUVECs migration assay using a Chemotaxicell chamber (Kurabo). The chambers were placed in a 24-well plate containing 500 µl HuMedia-EG2 with 0.1% FBS. HUVECs (1×10^5 cells) were incubated on the upper chambers for 30 min and treated with or without 18-deoxyherboxidiene for 1 h. After VEGF (3.1 ng ml^{-1}) stimulation, the cells were allowed to migrate from the upper to lower chamber for 9 h in 5% CO_2 at 37 °C. Migrated cells were fixed with CH_3OH, stained and counted. As shown in Figure 2a, 18-deoxyherboxidiene inhibited VEGF-induced HUVECs migration in a dose-dependent manner without significant cell toxicity, similar to SU5614, a well-known VEGFR2/PDGFR kinase inhibitor. Cell viability was measured by trypan blue assay (data not shown).

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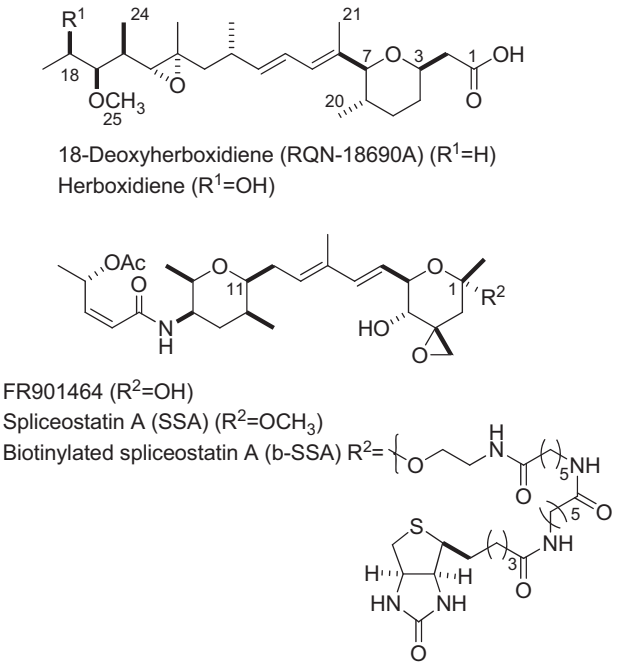

Figure 1 Chemical structures of 18-deoxyherboxidiene (RQN-18690A), herboxidiene, FR901464, spliceostatin A (SSA) and biotinylated SSA (b-SSA).

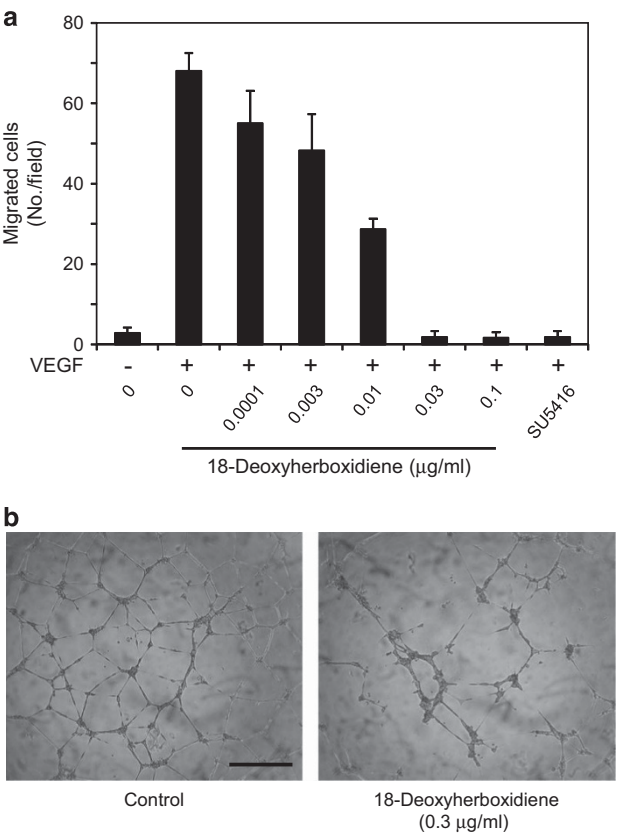

Figure 2 18-Deoxyherboxidiene inhibits the migration and tube formation of HUVECs. (**a**). 18-Deoxyherboxidiene inhibits VEGF-induced migration of HUVECs. SU5614, 10 μM. Each value represents the mean ± s.e. from three independent experiments. (**b**). 18-Deoxyherboxidiene suppresses capillary-like tube formation of HUVECs. Scale bar, 1 mm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

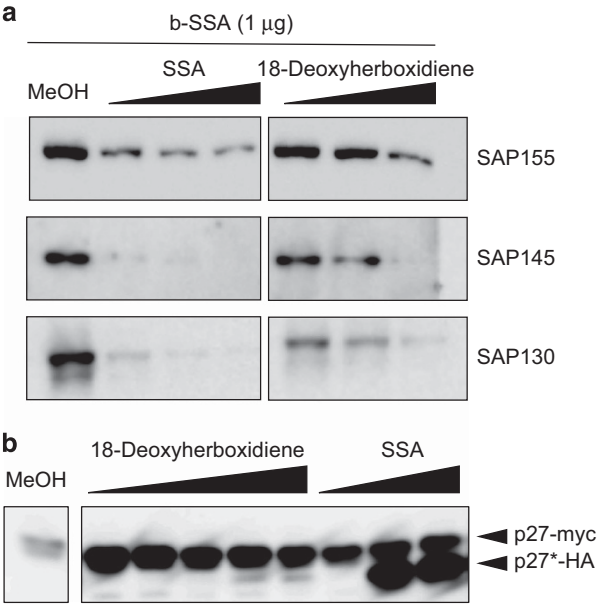

Figure 3 18-Deoxyherboxidiene targets SF3b, a spliceosome component *in vitro* and *in vivo*. (**a**) Competition assay by 18-deoxyherboxidiene against the binding of b-SSA to SF3b. SSA, 1, 10 and 100 ng. 18-deoxyherboxidiene, 100, 1000 and 10 000 ng. (**b**). Pre-messenger RNA translation in 18-deoxyherboxidiene-treated cells. SSA, 1, 10 and 100 ng ml^{-1}. 18-deoxyherboxidiene, 1, 10, 100, 1000 and 10 000 ng ml^{-1}.

We next performed a capillary-like tube formation assay on the Matrigel matrix (BD Biosciences, Bedford, MA, USA). HUVECs (1.5×10^4 cells) were treated with or without 18-deoxyherboxidiene for 16 h in 5% CO_2 at 37 °C and the capillary-like tubes were photographed under a phase contrast microscope. HUVECs significantly formed a capillary-like tube form, whereas 18-deoxyherboxidiene attenuated the vessel networks as well as the capillary-like tube formation, as shown in Figure 2b. These results suggest that 18-deoxyherboxidiene exhibits potent antiangiogenic effects in HUVECs.

Recently, spliceostatin A (SSA, a stable methyl-ketal derivative of FR901464),[17] pladienolide[18] and herboxidiene (GEX1A),[19] which have a reactive epoxide group, were shown to have a spliceosome inhibitory activity. SSA inhibits *in vitro* splicing and promotes pre-messenger RNA (mRNA) accumulation by binding to spliceosome-associated protein (SAP) 130 or SAP155, a subunit of SF3b in the spliceosome. Pladienolide and herboxidiene bind to SAP130 and SAP155, respectively. We therefore tested the effect of 18-deoxyherboxidiene on the binding to spliceosome using a biotinylated SSA (b-SSA). HeLa cell extracts (300 μg per assay) pretreated with or without 18-deoxyherboxidiene were incubated with b-SSA for 6 h at 4 °C.[17] After UltraLink immobilized streptavidin plus (Thermo Fisher Scientific, Waltham, MA, USA) was added, the mixture was further incubated for 1 h at 4 °C. The bound proteins were separated by SDS-PAGE and analyzed by western blotting analysis using anti-SAP130 (Abcam, Cambridge, UK), anti-SAP145 (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-SAP155 (Medical & Biological Laboratories, Nagoya, Japan) antibodies. As shown in Figure 3a, each SAP130, SAP145 and SAP155 subunit was detected as a b-SSA-binding protein, whereas 18-deoxyherboxidiene abolished this binding in a dose-dependent manner, similar to the effect of SSA, indicating that 18-deoxyherboxidiene targets SF3b.

We next examined the effect of 18-deoxyherboxidiene on pre-mRNA translation in p27-int-HA-transfected human embryonic kidney (HEK) 293 T cells, according to the previous report.[17] The p27-int-HA reporter system contains a hemagglutinin (HA) tag sequence before the in-frame termination codon in intron 1 and a Myc tag sequence before the termination codon in exon 2 in the gene encoding p27, a CDK inhibitor protein. This system enables us to detect p27-myc and p27*-HA proteins as proteins translated from correctly spliced or unspliced mRNA, respectively. p27-int-HA-transfected HEK 293 T cells treated with carrier solvent (MeOH) produced only the Myc-containing protein p27-Myc (Figure 3b). 18-Deoxyherboxidiene induced cells to generate the HA-tagged protein p27*-HA in addition to the p27-Myc form, although the induction effect of 18-deoxyherboxidiene on p27*-HA protein translation was weaker than that of SSA. These results suggest that 18-deoxyherboxidiene induces not only to accumulation of unspliced mRNA but also its translation to generate p27* by targeting SF3b.

SSA also blocks angiogenesis by inhibiting global gene expression including VEGF, exhibiting a potent antitumor activity.[20] Very recently, heroboxidiene was reported to attenuate tumor angiogenesis through the dual inhibition of VEGF-mediated signaling and hypoxia-inducible factor (*HIF*)-*1α* mRNA expression.[21] In our wound-healing assay, the inhibitory activity of 18-deoxyherboxidiene was ca. 10 times weaker than that of heroboxidiene (data not shown). Small molecules targeting SF3b would exert antiangiogenic activity, as structurally different herboxidienes as well as SSA has a strong antiangiogenic activity. Targeting the spliceosome by small-molecule inhibitors is emerging as a promising strategy to treat cancer by intercepting the splicing pathway.[22–24] This splicing process is catalyzed by the spliceosome, which is composed of five snRNPs (U1, U2, U4, U5 and U6) and over 150 associated proteins.[25] In particular, SF3b, a subcomplex of the U2 snRNP is a common target for splicing inhibitors such as SSA, pladienolide, herboxidiene and also 18-deoxyherboxidiene.

In summary, we have identified RQN-18690A (18-deoxyherboxidiene) from *Streptomyces* sp. QN18690 as a promising angiogenesis inhibitor, and revealed that 18-deoxyherboxidiene targets SF3b both *in vitro* and *in vivo*. Further structure–activity relationship studies on 18-deoxyherboxidiene as a spliceosome inhibitor would contribute to the development of a new effective anticancer drug.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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