Original Article

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Both triazolyl ester of ketorolac (15K) and YM155 inhibit the embryonic angiogenesis *in ovo* (fertilized eggs) *via* their common PAK1-survivin/VEGF signaling pathway

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Summary **15 K is 1,2, 3-triazolyl ester of ketorolac, an old pain-killer, that blocks PAK1 by its R-form and inhibits COX-2 by its S-form. Mainly due to a robust increase in cell-permeability, 15K is over 500 times more potent than ketorolac in both anti-cancer and anti-PAK1 activities in cell culture with IC_{50} around 24 nM. However, 15K has no anti-AKT activity. Angiogenesis requires at least the kinase PAK1, and perhaps the kinase AKT as well, and is essential for a robust growth of solid tumors. Thus, in this study, we examined the potential antiangiogenic activity of 15K both *in ovo* and cell culture, prior to its *in vivo* (xenograft) anti-cancer activity test. The IC_{50} of 15K against the embryonic angiogenesis *in ovo* in CAM (chorioallantoic membrane) assay is around 1 nmol/egg. Surprizingly, however, 15K failed to inhibit the tube formation of HUVECs (human umbilical vein endothelial cells) in cell culture even at high as 150 μM. In an attempt to solve this mystery, we tested both *in ovo* as well as HUVECs-based anti-angiogenic activity of a potent survivin-suppressor called YM155, which blocks PAK1, in addition to AKT. YM155 is slightly more potent than 15K in CAM assay with IC_{50} around 0.5 nmol/egg, and apparenty inhibits the tube formation of HUVECs with IC_{50} around 18 nM. According to a few previous findings with the direct PAK1-inhibitor frondoside A (FRA), the tube formation of HUVECs depends solely on PAK1. Thus, the failure of 15K to affect their tube formation is most likely due to their drug (15K)-resistance. Furthermore, unlike FRA, YM155 killed HUVECs with IC_{50} around 18 nM, clearly indicating that AKT is essential for survival of HUVECs, instead of their tube formation.**

Keywords: PAK1, angiogenesis, HUVECs, ketorolac ester (15K), YM155, survivin suppressor

1. Introduction

PAK1 (RAC/CDC42-activated kinase 1) is the major oncogenic/ageing/melanogenic kinase. It is responsible for a wide variety of diseases/disorders such as cancers, neurofibromatosis (NF), Alzheimer's disease (AD), diabetes (type 2), hypertension, a variety of infectious and inflammatory diseases, epilepsy, schizophrenia, depression, autism, and obesity (*1*). In addition, PAK1 shortens the healthy lifespan of *C. elgans* (*2*), and is essential for PDGF/EGF-dependent melanogenesis as well (*3*), suggesting that PAK1-blockers could be elixirs (longevity-promoters) and skin-whitening cosmetics. Thus, the potential market value of natural or synthetic PAK1-blockers would be huge, and pharmaceutical giants such as Pfizer, Roche, Novartis and Astrazeneca recently started developing potent PAK1-blockers.

However, major problem of these synthetic PAK1-blockers for clinical application is their poor water-solubility and cell-permeability. Thus, through the copper-catalyzed "Click Chemistry" (CC) which was originally introduced by Barry Sharpless (2001 Nobel-laureate) and his colleagues in 2001 (*4*), we recently managed to robustly boost both anti-cancer activity and cell-permeability of several COOH-bearing PAK1-

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blockers without any loss of their water-solubility (*5*). Briefly, any COOH-bearing compounds could be esterized with water-soluble 1,2,3-triazolyl alcohol in a high yield through the CC. Among these 1,2,3-triazolyl esters, 15K, 1,2,3-triazolyl ester of an old pain-killer called ketorolac sold by Roche, is so far the most potent, inhibiting the PAK1/COX-2-dependent growth of A549 lung cancer cells with IC_{50} around 24 nM, and the growth of B16F10 melanoma cells with IC_{50} around 6 nM (*5*). In addition, by the CC, the anti-PAK1 activity of ketorolac in cell culture was boosted over 500 fold, and the anti-COX-2 activity *in vitro* was boosted 20 fold, respectively (*5*).

Angiogenesis, which is essential for the robust growth of all solid tumors, depends on PAK1 (*6*). It has been known that the oncogenic RAS-PAK1-RAF-MEK-ERK signalling pathway leads to activation of VEGF (vascular endothelial growth factor) gene which is essential for angiogenesis (*7*). Prior to its test *in vivo* (xenograft) anti-cancer test, we wonder if ketorolac or its very potent derivative (15K) is anti-angiogenic. However, so far there is no positive report on the anti-angiogenic activity of Ketorolac *per se* in cell culture or *in vivo*. Here in this study, we have confirmed the potent anti-angiogenic activity of 15K *in ovo* by chorioallantoic membrane (CAM) assay (*8*), although it has no effect on the tube formation (or survival) of HUVECs in cell culture. We wonder why.

More interestingly, we recently found that YM155, a potent survivin-suppressor, also blocks PAK1 (in addition to another oncogenic/angiogenic kinase AKT) in cell culture (*9*). This is not a great surprise because it has been known that PAK1-deficient mice express far less survivin, clearly indicating that PAK1 is essential for survivin expression (*10*). Interestingly, however, YM155 blocks AKT by inhibiting epithelial growth factor (EGF) receptor (*11*). Thus, in an attempt to solve the mystery behind the inability of 15K to block the tube formation of HUVECs, we examined if YM155 affects the *in ovo* anti-angiogenesis as well as both tube formation and viability of HUVECs in cell culture (*8,12*), as their viability might depend on AKT, instead of PAK1.

2. Materials and Methods

2.1. *Chemicals and reagents*

15K, 1,2,3-triazolyl ester of ketorolac (see Figure 1A), was synthesized from ketorolac by Click Chemitsry as described previously (*5*). YM155 (see Figure 1B) was purchased from Adooq Bioscience (Irvine, CA, USA). Medium MCDB-104 was a product of Nihon Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Moregate (Brisbane, Australia). Cellgen was obtained from Koken (Tokyo, Japan). EGF was purchased from BD Biosciences

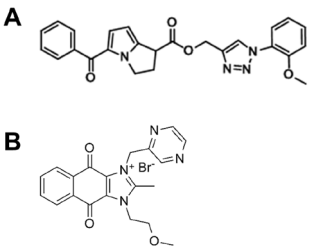

Figure 1. Chemical structure of 15K (A) and YM155 (B).

(Bedford, MA). Human basic fibroblast growth factor (FGF, recombinant) was purchased from Austral Biologicals (San Ramon, CA, USA). Medium 199 and all other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Fertilized chicken eggs were obtained from Pulmuone Farm (Danyang, Korea). Fat emulsion (20%) was from DongKook Pharmaceutical Co., Ltd. (Seoul, Korea).

2.2. *Cell culture*

HUVECs were grown in HUVEC growth medium (MCDB-104 medium supplemented with 10 ng/mL EGF, 100 µg/mL heparin, 100 ng/mL endothelial cell growth factor and 10% FBS) as previously described (*8*). Cell culture was carried out at 37°C under a humidified 95-5% (v/v) mixture of air and CO_2. The cells were seeded on plates coated with 0.1% gelatin and allowed to grow to subconfluence before experimental treatments.

2.3. *CAM angiogenesis assay*

The CAM assay was performed as previously described (*12,13*). In brief, fertilized chicken eggs were kept in a humidified incubator at 37°C. After 4 days of incubation, approximately 4 mL of albumen was aspirated and further incubated. Onto 5-day-old fertilized chicken embryos in the shells, 10 µL aliquots of samples (0.2, 1, 5, and 20 nmol/egg) or retinoic acid (5 nmol/egg), as a positive control mixed in 1% methylcellulose, were applied in 2 mm silicon rings placed on the surface of the growing CAM. After 2 days of incubation, an appropriate volume of a 20% fat emulsion was injected into the CAM to visualize the blood vessels. At least 15 eggs were used for each condition, and experiments were repeated 5 times. The % inhibition of angiogenesis by 15K or YM155 was calculated as a suppression ratio of new vessels within the area encircled by a white ring, compared with the control (non-treated).

2.4. *HUVECs tube formation assay*

Capillary tube-like structures formed by HUVECs were prepared as previously described with slight modifications (*8,12*). Briefly, HUVECs (6.0×10^4 cells/

cm^2) were seeded between two layers of collagen gel and then incubated in MCDB-104 medium with 0.5% FBS supplemented with 10 ng/mL of basic fibroblast growth factor, 8 nM phorbol 12-myristate 13-acetate, and 25 µg/mL ascorbic acid. They were treated with various concentrations of either 15K (50 and 150 µM), YM155 (1, 5, 10, 25, and 50 nM) or CAPE (50 µM) for up to 24 h. The resulting web-like capillary structure was viewed with a microscope under 200 x magnification and captured with a Leica-DFC295 digital camera (Leica, Wetzlar, Germany). Based on these photographs, the tube formation was quantified by determining the pixel number of tubes in each image using the NIH Image program.

2.5. *HUVECs viability during YM155 treatment*

The cell viability of HUVECs was determined by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) method as previously described (*3,5*). In brief, HUVECs (1.0×10^4 cells/cm^2) were seeded and then treated as above with YM155 at indicated concentrations (1-50 nM) for 24 h. Their viability was determined with MTT reagent which is converted to a pigment by a mitocondrial reductase in living cells. The absorbance (OD) of this pigment was measured at 490 nm, using a microplate absorbance reader (iMark™, Bio-Rad Laboratories, CA, USA).

2.6. *Statistical analysis*

Results are expressed as mean ± SD (standard deviation) from five independent experiments. A value of $p < 0.05$ was considered statistically significant. Data were evaluated statistically using one-way analysis of variance (ANOVA) followed by Holm-Sidak method and using Student *t*-test for analysis between the control and treatments (15K and YM155).

3. Results

3.1. *Suppression of embryonic angiogenesis in ovo by 15K and YM155*

To evaluate the effect of 15K (for chemical structure, see Figure 1A) and YM155 (for chemical structure, see Figure 1B) on the angiogenesis, we first used a CAM assay, an increasingly popular *in ovo* model for studying angiogenesis which was adapted by Folkman in an early 1970s (*14*). In CAM assay, anti-angiogenic activities of either 15K or YM155 were judged on 7th day after 2 days treatment, and retinoic acid (RA, 5 nmol/egg) was used as a positive (anti-angiogenic) control (*13,15*). As shown in Figure 2, 15K significantly inhibited the new blood vessel growth of chick embryos in a dose-dependent manner (0.2-20 nmol/egg). The IC_{50} of 15K against the embryonic angio-genesis was around 1 nmol/egg. At 5 nmol/egg, 15K and RA showed the basically same level of anti-angiogenic effect, inhibiting the angiogenesis by around 65%.

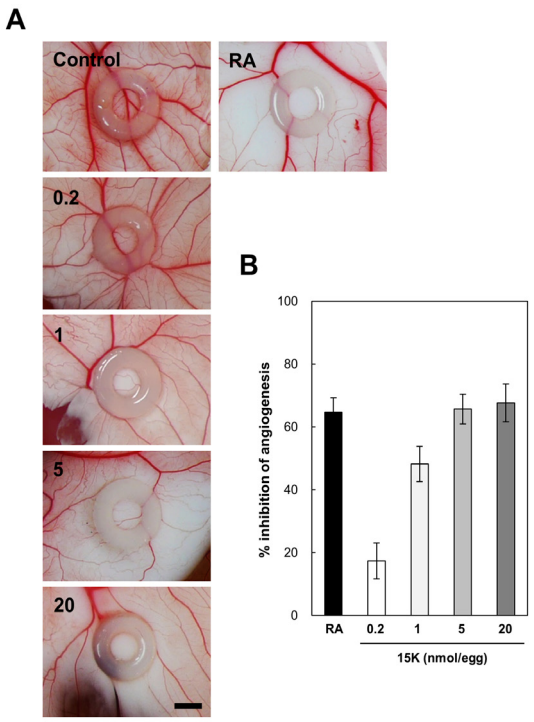

Figure 2. Anti-angiogenic activity of 15K *in ovo*. A. *in ovo* angiogenesis patterns: the control (non-treated) embryo angiogenesis, treated with either 15K (0.2-20 nmol/egg) or retinoic acid (RA, 5 nmol/egg). **B**. Quantification (% inhibition of angiogenesis), based on the new vessel formation within the area encircled by the white ring (compared with the control). 15K significantly inhibited the angiogenesis in CAM in a dose-dependent manner with IC_{50} around 1 nmol/egg. Scale bar = 2 mm. Values are expressed as means ± SD ($n = 5$).

As shown in Figure 3, YM155 also showed a strong anti-angiogenic activity *in ovo* in a dose-dependent manner, with IC_{50} around 0.5 nmol/egg, suggesting that YM155 appears to be even more potent than 15K under the CAM conditions. Interestingly, IC_{50} of YM155 against PAK1 in cell culture is around 500 nM, being 10 times higher than that of 15K (around 50 nM), and it is also true with anti-productivity (reducing brood size) and elixir (life-extending) activity in *C. elegans* (*9*). These observations strongly suggest, if not proven as yet, that the *in ovo* anti-angiogenesis of YM155 is not only due to its anti-PAK1 activity, but also due to its anti-AKT activity (or a third unknown activity). Is there any evidence for AKT-dependency of *in ovo* angiogenesis? Recently it has been suggested that AKT is involved in the COX-2-dependent *in ovo* angiogenesis (*16*).

3.2. *Effect of 15K and YM155 on HUVEC tube formation*

We next investigated the effect of 15K and YM155 on the angiogenesis *in vitro* (cell culture) using a tube formation model of HUVECs, in comparison with

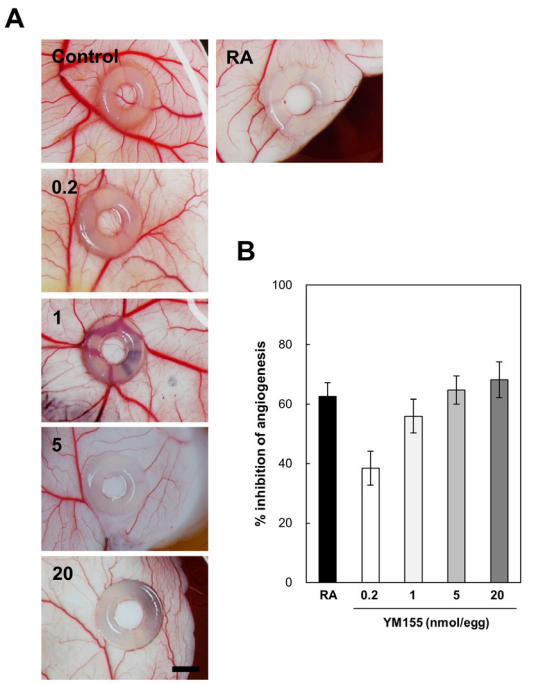

Figure 3. Anti-angiogenic activity of YM155 *in ovo*. A. *in ovo* angiogenesis patterns: the control (non-treated) embryo angiogenesis, treated with either YM155 (0.2-20 nmol/egg) or Retinoic acid (RA, 5 nmol/egg). **B**. Quantification (% inhibition of angiogenesis). YM155 also inhibited the *in ovo* angiogenesis in CAM in a dose-dependent manner with IC_{50} around 0.5 nmol/egg. Scale bar = 2 mm. Values are expressed as means ± SD (n = 5).

caffeic acid phenethyl ester (CAPE), an anti-angiogenic PAK1-blocker from propolis. During normal tube formation, the endothelial cells formed a network of capillary-like tubes, which were composed of multiple cells by gathering together and adhering to each other.

As shown in Figure 4D, YM155 clearly inhibited their elongation with IC_{50} around 18 nM. CAPE at 50 μM also completely inhibited their elongation, with around 50% cell death (see Figure 4B). To our big surprise, however, 15K, even at 150 μM caused little effect on tube formation (or survival) of these endothelial cells (see Figure 4B).

The IC_{50} of 15K and CAPE against the PAK1-dependent growth of A549 lung cancer cells for 72 h are 24 nM and 10 μM, respectively, indicating that 15K is over 400 fold more potent than CAPE, if both are equally allowed to penetrate through plasma membranes of their target cells. Thus, in theory, 15 K should have inhibited their tube formation for 24 h with IC_{50} around 120 nM, if 15K is allowed to penetrate as effectively as CAPE through HUVECs. In short, it is most likely that 15K fails to penetrate through the plasma membranes of HUVECs.

Is there any evidence for AKT-dependency of HUVEC tube formation? Both AKT and ERK (down stream of PAK1) signaling pathways have been reported to be involved in the HUVEC angiogenesis (*17*). Furthermore, the observed IC_{50} of YM155 (18 nM) aginst HUVEC tube formation is not far from the IC_{50} against the oncogenic EGFR-PI-3 kinase-AKT/ERK signalig pathway in pancreatic cancer cells (*11*). In addition, it should be worth noting that CAPE, which clearly inhibits the tube formation of HUVECs, is known to block AKT, in addition to MAPK/ERK, down-stream of PAK1 (*18*), as does YM155.

3.3. *Cell death of HUVECs during YM155 treatment*

CAPE at 50 μM caused around 50% death of HUVECs. We observed a significant % of their death during YM155 treatment as well. Thus, in an attempt to distinguish between cell death and *bona fide* inhibition of tube formation *per se*, we have quantified their YM155-caused death (see Figure 5), an an attempt to re-calculated the latter (net inhibition of tube formation), according to the formula as discussed below. Up to 10 nM, YM155 inhibited the *bona fide* tube formation by 20-30% (presumably due to its anti-PAK1 activity), but killed HUVECs by its anti-AKT activity with IC_{50} around 18 nM.

4. Discussion

First of all, these *in ovo* observations altogether have proven that both 15K and YM155 exert their potent anti-angiogenic activity by blocking their common oncogenic/angiogenic PAK1-survivin signaling pathway, as PAK1 is essential for survivin expression (*10*), and the potent survivin-suppressor YM155 indeed blocks PAK1 as well as AKT in cancer cells (*9,11*). To the best of our knowledge, both 15K and YM155 are the most potent anti-angiogenic PAK1-blockers so far. Moreover, since we recently found that 15K extends the healthy lifespan of *C. elegans* by 30% at 50 nM (*15*) as does PAK1-deficiency (*1,2*), it is most likely that 15K is also able to suppress effectively the angiogenesis-dependent growth of a variety of solid tumors such as pancreatic and colon cancers *in vivo* (for instance, human cancer xenografts in mice) without any side effect, as does YM155. Currently we are testing the effect of 15K on the growth, metastasis and angiogenesis of human cancer xenografts in mice.

In addition, it should be worth warning that the simple HUVECs cell culture system turns out to be a rather imperfect model to screen for some potent anti-angiogenic compounds such as 15K and retinoic acid which work *in ovo*, but clearly fail in this mono-cell culture, most likely due to their faillure to penetrate through plasma membrane of HUVECs. Ultimately siRNA-based AKT/PAK1 silencing approach should determine whether the tube formation of HUVECs depends on AKT or PAK1.

However, it should be worth proposing the following (far less expensive and quicker) alternative approach: a highly cell-permeable potent anti-cancer

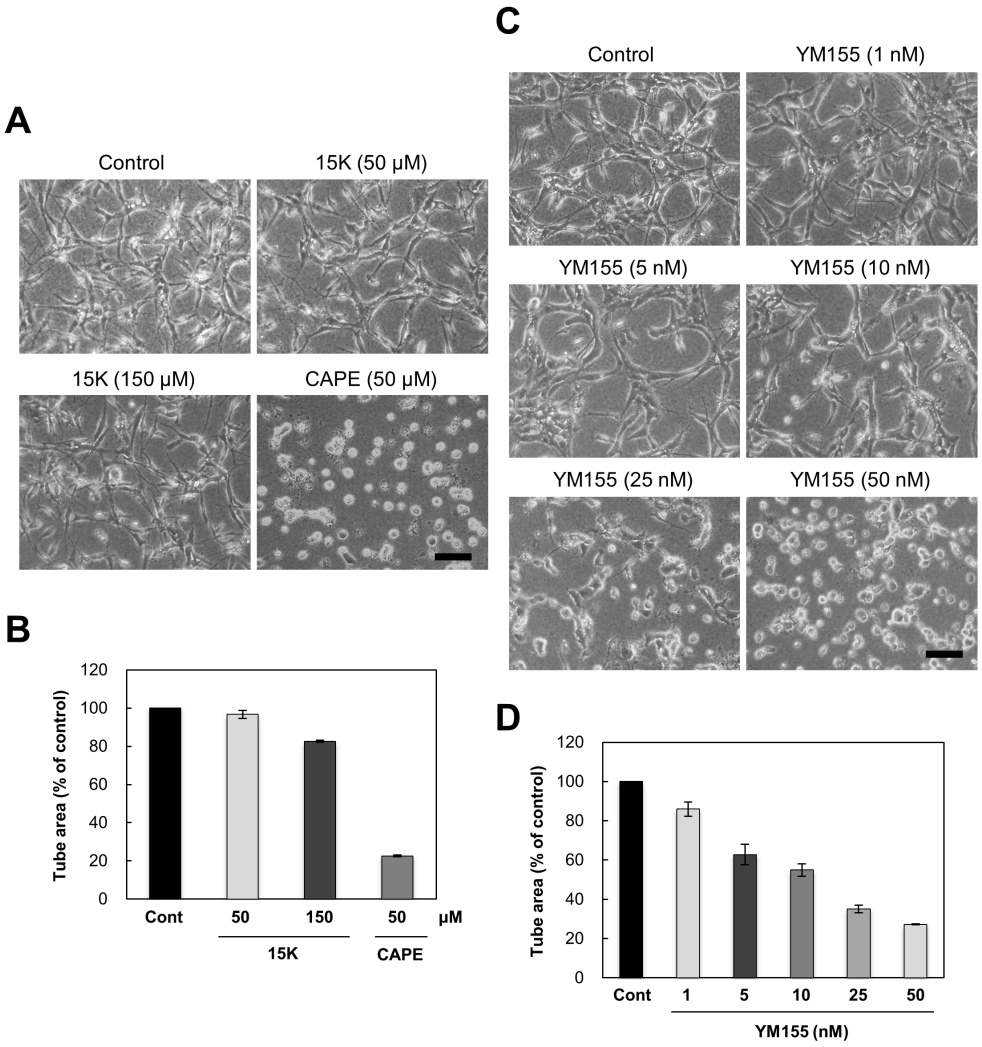

Figure 4. YM155 (C, D) inhibits the tube formation of HUVECs, but not 15K (A, B). HUVECs were sandwiched between two layers of collagen gels and induced to form blood vessel-like tubes. **Left**: HUVEC were treated with 15K at indicated concentrations (0, 50 and 150 μM). **The quantification (B)**: % tube area was measured by the length of tube-like structures containing connected cells. 15K failed to inhibit tube formation of HUVECs. **Right**: HUVECs were treated with YM155 at indicated concentrations (0, 1, 5, 10, 25, and 50 nM). **The quatification (D)**: YM155 apparently inhibits the tube formation in a dose-dependent manner, with IC50 around 18 nM. Each experiment was repeated three times and representative data are shown. Scale bar = 100 μm.

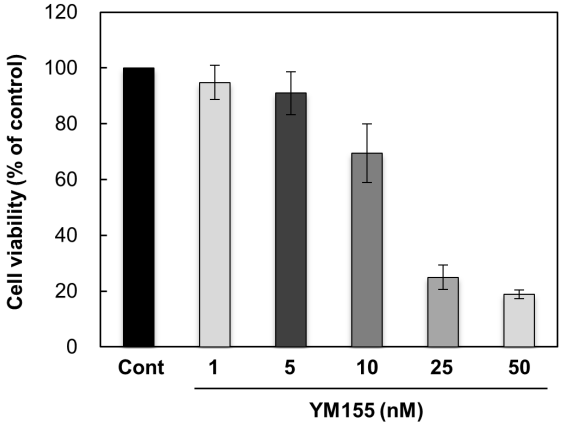

Figure 5. Cell viability of HUVECs during YM155 treatment. HUVECs were treated with YM155 at the indicated concentrations for 24 h, and their viability was measured by MTT method. YM155 reduced their viability in a dose-dependent manner with IC_{50} around 18 nM. Each experiment was repeated three times.

agent called frondoside A (FRA), a sulphated saponin, from a sea cucumber directly inhibits PAK1 with IC_{50} around 1 μM, and AKT with IC_{50} around 60 μM (*19*). If FRA fails to inhibit the tube formation around at 1 μM, but works around at 60 μM, it would be crystal clear that (unlike *in ovo* angiogenesis) the tube formation of HUVECs is AKT-dependent, and PAK1-independent, as is the survival of all normal cells. To a big surprise, however, the outcome is clearly opposite: their tube formation requires PAK1, while their survival requires AKT (see Figure 6). FRA has been found to inhibit the tube formation of HUVECs in cell culture by more than 80% at 0.5 μM without any cytotoxicity, proving that its anti-PAK1 activity alone is sufficient for its anti-angiogenic action against HUVECs (*20*). Thus, we shall conclude that the potent PAK1-blocker 15K fails to interfere with the tube formation of HUVEC simply by its failure in penetrating through this cell line, just like

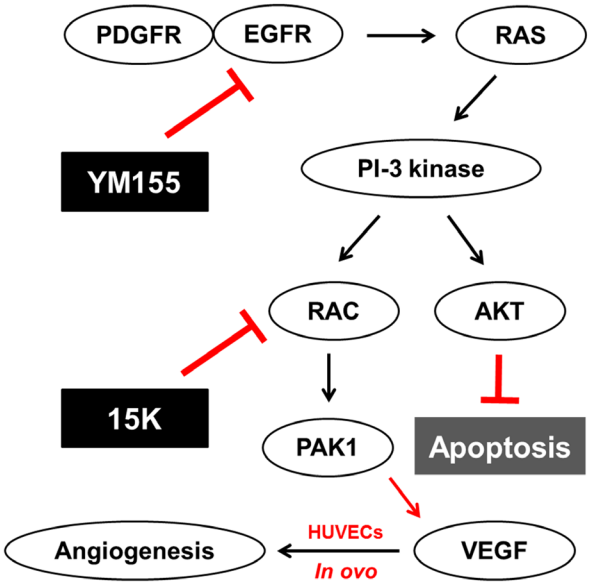

Figure 6. Mechanism underlying the anti-angiogenic action of 15K and YM155 *in ovo* and cell culture. 15K blocks PAK1 by inhibiting RAC directly, and inhibiting COX-2 directly, down stream of PAK1. YM155 blocks both PAK1 and AKT by down-regulating EGFR, and eventually suppressing survivin and COX-2 genes. EGFR-RAS-PI3 kinase-PAK1-MEK-ERK signalling pathway eventually activates VEGF gene. Thus, if *in ovo* angiogenesis depends on either PAK1 or AKT (or both), both 15K and YM155 could inhibit the embryonic angiogenesis. So far it is most likely that tube formation of HUVECS depends solely on PAK1 (*19,20*), while their survival depends mainly on AKT (*21*). Thus, the most likely reason why YM155 causes their death and inhibits their tube formation, but 15K fails is that HUVECs simply rejects 15K.

a few other PAK1-dependent cancer cell lines such as EMT6 (breast) and LIM-1899 (colon) (*5*).

In additon, we should point out another pitfall of HUVECs-based tube formation system. Many normal cells including HUVECs require AKT, but not PAK1, for their survival (*1,20,21*). Thus, any compounds with anti-AKT activity are expected to kill HUVECs at critical concentrations. In fact CAPE at 50 μM killed around 50% of HUVECs. Since the dead HUVECS would no longer form tubes, and the apparent reduction of tube formation by CAPE at this concentration is around 80%, the net inhibition of tube formation should be corrected to only 30% of total cells. In other words, the anti-PAK1 activity of CAPE inhibited the 60% of the remaining (50%) cells. Likewise, the apparent % reduction in tube formation (a) by YM155, which also kills HUVECS by its anti-AKT activity (see Figure 5), should be corrected by the following formula where their % death is b: (a – b)/(100 – b) × 100%. Thus, if a = b, in fact there is no net inhibition of tube formation *per se*. According to the above formula, up to 10 nM, the *bona fide* inhibition of tube formation by YM155 reached 20-30%, but at 25 nM (and higher concentrations), the cell death by its anti-AKT activity dominated.

Unfortunately, many scientists tend failing to distinguish between their death and *bona fide* inhibiton of tube formation, drawing an apparently conflicting (or incorrect) conclusion on PAK1/AKT-dependency of angiogensis in general.

In conclusion, due to the MDR (multi-drug resistance) of HUVECs, their tube formation assay appears to be a less reliable model for angiogenesis, compared with *in ovo* CAM assay. Furthermore, just like the case of CAPE and YM155, the apparent inhibition of tube formation is often caused by cell death (apoptosis), rather than the inhibition of cell migration and attachment *per se*. Despite of these pitfalls, as long as test compounds could permeate HUVECs without cell death as does FRA (*20*), the tube formation could provide an alternative cell culture screening system for PAK1-specific blockers, as is the PDGF-dependent melanogenesis without cell death in B16F10 melanoma cell line (*3*).

Lastly, 15K appears to be a far safer cancer therapeutic than YM155, mainly because it causes no harm on normal cells such as HUVECs which are clearly killed by YM155.

Acknowledgements

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