1,2,3-Triazolyl ester of ketorolac (15K): Boosting both heat-endurance and lifespan of *C. elegans* by down-regulating PAK1 at nM levels

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### Summary

PAK1 (RAC/CDC42-activated kinase 1) is the major oncogenic/ageing kinase, and its dysfunction extends the healthy lifespan of *C. elegans* by activating *HSP16* gene. 15K is a highly cell-permeable 1,2,3-triazolyl ester of ketorolac that down-regulates both PAK1 and its down-stream COX-2 in R- and S-forms, respectively. 15K is 500-5,000 times more potent than ketorolac, an old pain-killer, inhibiting the growth of cancer cell lines with IC₅₀ ranging 5-24 nM. Scores of natural and synthetic PAK1-blockers have been shown to extend the lifespan of small animals such as *C. elegans*, but none of them has been effective at nM levels. Thus, we examined in vivo effect of 15K at nM levels on the survival rate of *C. elegans* with or without heat-shock. Like the PAK1-deficient mutant, 15K (at 50 nM)-treated worm significantly lives longer, is far more heat-resistant and less productive (fertile) than the non-treated counterpart, with an increased expression of *HSP16* gene. 15K has been proven to be among the most potent anti-cancerous and longevity-promoting PAK1-blockers, and therefore has a potential to treat a variety of solid tumours without severe side effect.

**Keywords:** Ketorolac, 1,2,3-triazolyl ester, PAK1, *C. elegans*, lifespan, anti-cancer

### 1. Introduction

PAK1 is a Ser/Thr-kinase that is activated by the GTPases (RAC/CDC42), and essential for the growth of over 30% human cancer called RAS cancers which carry the oncogenic mutant of Ki-RAS (1-3). This "RAS cancer" represent over 90% of pancreatic cancer, 50% of colon cancer and 30% of lung cancer (1). Furthermore, since solid tumours require PAK1-dependent angiogenesis for their robust growth (2-4), it is most likely that the majority of other solid tumours also depends on PAK1. Interestingly PAK1 is not essential for normal cell growth (2,3). Furthermore, PAK1-deficient mutant (RB689) of *C. elegans* lives 60% longer than the wild-type, clearly indicating that PAK1 is among the major ageing kinases shortening the healthy lifespan (5). Thus, in principle, unlike all conventional anti-cancer drugs (DNA/RNA/microtubules poisons), none of highly-specific PAK1-blockers are expected to cause any severe side effects. In fact scores of natural PAK1-blockers and even a few synthetic ones have been shown to extend the lifespan of small animals such as *C. elegans*, but so far none of them has been effective at nM levels (6).

We recently developed a potent PAK1-blocker called 15K from Ketorolac, an old pain-killer via Click Chemistry (7). 15K is a highly cell-permeable 1,2,3-triazolyl ester of ketorolac (Figure 1), a racemic COOH-bearing PAK1-blocker/COX-2 inhibitor, which down-regulates PAK1 in R-form, as well as directly inhibits COX-2 in S-form (7,8). 15K is over 500-5,000 times more potent than ketorolac to inhibit the growth of cancer cells such as A549 (lung) and B16F10 (melanoma) cell lines with IC₅₀ ranging 5-24 nM (7).
Furthermore, 15K inhibits the embryonic angiogenesis in ovo (fertilized eggs) with IC_{50} around 1 nmol/egg (9).

In this study, prior to in vivo (human cancer xenograft in mice) test of 15K, we examined whether 15K (10-100 nM) could extend (or shorten) the healthy lifespan of C. elegans which has the shortest mean lifespan (around 2 weeks) among experimental animals, as did either the PAK1-KO (knock-out) or a herbal PAK1-blocker called triptolide (around 140 μM) previously (4,10), in an attempt to confirm that 15K causes little side effect on this worm at least.

2. Materials and Methods

2.1. Strain of C. elegans and reagents

The strain CL2070 of C. elegans was kindly provided by CGC (C. elegans Genomic Center). 15K was synthesized from ketorolac via Click Chemistry as previously described (7).

2.2. Measurement of brood size

The wild-type (N2) of C. elegans was fed by the lawn culture of E. coli (OP50) which was grown in the presence or absence of 15K at 25, 50 and 100 nM on the standard NGM (nematode growth medium) agar plate for 2 days shortly after the hatching at 23°C. Then the number of eggs laid by each group of around 40 adult worms overnight (for 10 hours) was counted. The brood size was calculated as the number of eggs per mother (female worm).

2.3. Survival of worms after prolonged heat-shock treatment

The wild-type (N2) was fed by E. coli which was grown in the presence or absence of 10-100 nM of 15K for 2 days as described above. Then each group of 60 adult worms was heat-shocked at 35°C for 8 hours. Then each group was cultured at 20°C for over 2 weeks. Every day after the heat challenge, the number of "dead" worms in each group was counted for scoring their survival rate. For statistical analysis, we employed the log-rank analysis (11).

2.4. HSP16.2 dependent GFP expression

The strain CL2070 which carries a transgenic reporter gene called "HSP16.2-GFP" fusion gene (12) was fed by E. coli (OP50) which was grown in the presence or absence of 25-100 nM of 15K overnight (12 hours) at 22°C. Then each group of around 20 worms was heat-shocked at 35°C for 2 hours, and then kept at 22°C for the recovery. After 4 hours, each group was fixed with a drop (10 micro liter) of sodium azide (1 M) on slides for microscopy. Under blue light which stimulates the green florescence emission from GFP produced in each worm, the fluorescence images were acquired at the same exposure parameters, using a 40X objective of the microscope (BX60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; QImaging, Burnaby, British Columbia, Canada).

2.5. Measurement of lifespan

Five L4/young adult worms were transferred to a fresh NGM plate and permitted to lay eggs for 5 hours. After removing the five adult worms, the progeny were grown on NGM plates for 3 days. The lifespan of the age-synchronized hermaphrodites (5,10,13) at 20°C was measured on the agar plates with the lawn culture of the control E. coli (OP50) or those cultured with 15K at 10-100 nM, as we have done with triptolide (140 μM) previously (13). In order to prevent progeny production, 5-fluoro-2’-deoxyuridine (F UdR; Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the agar plate at the final concentration of 36 μM after the animals had reached adulthood as described previously (5,10,13). The log-rank test was employed for statistical analysis (11).

2.6. Statistic analysis

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by one-way ANOVA. Statistical analysis was conducted using SPSS (release 16.0, Chicago, Illinois) and p < 0.05 was considered significant.

3. Results and Discussion

3.1. 15K reduces the brood size (number of eggs laid) of C. elegans

In the past, either PAK1-KO (knock-out) or treatment with herbal PAK1-blockers such as CAPE (caffeic acid phenethyl ester) reduced number of eggs laid by female worms, while it extended their healthy lifespan, indicating that there is a clear "trade-off" relationship between fertility and lifespan (2,5). Since fertility assay takes only a few days, while lifespan assay takes over a month, we first tested the effect of 15K on the number of eggs laid, in order to determine the effective doses of 15K. As shown in Figure 2, 15K at 50-100 nM reduced their fertility by 60-70% in a dose-dependent manner.
suppressed by PAK1 (2,5,12). As shown in Figure 3A, 15K (10-100 nM) treatment of this worm increased its heat-resistance (survival rate after heat-shock at 35°C for 8 hrs) by several times. As expected, GFP expression under the control HSP16.2 gene promoter was also up-regulated with 15K (25-100 nM) treatment by 20-30% (see Figure 3B) after a brief (2 hrs) heat shock. Without heat-shock, however, no GFP was expressed (data not shown) as shown previously (5,12).

3.3. 15K extends the health lifespan of C. elegans

The mean lifespan of the control worm is around 18 days as shown in Figure 4 (open circles). However, 15K treatment of this worm at 50 nM clearly increased the healthy lifespan to around 21 days by around 15% (see Figure 4, closed circles), as does the natural PAK1-blocker "triptolide" (140 μM) treatment (13). However, 10 nM 15K showed no effect on the mean lifespan of this worm (data not shown).

As mentioned previously (7,8), racemic 15K has at least two direct targets: (i) R-form directly inhibits the GTPase RAC that activates PAK1, and (ii) S-form directly inhibits COX-2 whose expression depends on PAK1. Our present test concerning the effect of 15K on C. elegans is basically same as phenotypes of PAK1 KO (5) as well as effect of natural PAK1-blockers such as propolis and triptolide (13,14), confirming that the down-regulation of both PAK1 and COX-2 by 15K leads to a significant extension, instead of shortening, of its lifespan.

The lifespan-extending effect of "synthetic" chemicals has been very rarely tested on C. elegans, although so many "natural" PAK1-blockers were tested with clearly positive outcome (6). The most likely reason appears to be at least in part due to a "myth" or "chemo-phobia" that, unlike natural compounds, synthetic chemicals in general would be rather harmful to living things. However, there is an exception:
metformin, an old synthetic anti-diabetic/anti-obesity compound was shown to extend the lifespan of *C. elegans* at 50 mM (15). Here in this study, we have presented a second exception with 15K, and the effective dose of 15K is 1 million times lower than that of metformin. Even compared with potent natural PAK1-blocking elixirs such as CAPE (ED = 100 μM) and triptolide (ED = 140 μM), 15K is over 1,000 times more potent as an elixir (longevity-promoter) than these natural PAK1-blockers (6,13-16).

In this context, we would like to encourage molecular oncologists or chemotherapists working on synthetic PAK1-blockers/inhibitors to test their lifespan-extending effect on short-lived tiny animals such as *C. elegans* and *Drosophila*, prior to either *in vivo* cancer xenograft in mice or clinical trials for cancer therapy, making it sure that each synthetic chemical causes no harm to these tiny invertebrates at least.

Very recently a Chinese team reported that even an old semi-synthetic pain-killer called "Aspirin" at 100 μM also extends the lifespan of this worm in association with an increased heat-resistance (17). Furthermore, a new synthetic chemical called NPI that causes diet restriction also extends the lifespan of this worm at 50 μM (18). Thus, one could easily anticipate that even Ketorolac, an old synthetic pain-killer, which is 500 times less potent than 15K, also might extend the healthy lifespan of this worm at around 25 μM, although so far nobody appears to have tested its "elixir" (longevity-promoting) potential as yet.

It should also be worth pointing out that our assay for protection by 15K against pre-mature death of this worm after the prolonged heat-shock (equivalent to its protection against "global warming" effect) appears to be far more sensitive (and time-saving) than the standard assay for its life extending effect without heat-shock (compare Figure 3A and Figure 4). Thus, the former assay would be the better option (than the latter) for *in vivo* screening for PAK1-blockers such as 15K.

Lastly, since unlike vertebrates such as mice, *C. elegans* is among invertebrates that lack cardiovascular system, it would be worth testing the longevity-promoting effect of 15K in the shortest-lived vertebrate called African turquoise killifish as well, whose mean lifespan (around 4 months) could be extended by 50% by a natural PAK1-blocker called resveratrol at 2.5 μM (19). In general, up-regulation of PAK1 causes both hypertension and cranial hemorrhage in vertebrates such as zebra fish, and its down-regulation rescues the hypertension and hemorrhage (2,20), in favour of their longevity.

In conclusion, 15K is among the most potent synthetic PAK1-blockers as well as longevity-promoters, and it is most likely that 15K could be useful for treating a variety of solid tumors including neurofibromatosis (NF) as well as a series of many other PAK1-dependent diseases/disorders such as Alzheimer’s disease (AD) and hypertension without severe side effect. We are currently testing this notion *in vivo* (in mouse models), prior to clinical trials.

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**References**


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