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Mini Review

PAK1-Blockers (Natural and Synthetic) that Promote the Longevity and Heat-Endurance

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Abstract

In the past several distinct "ageing" genes have been identified in small animals such as *Caenorhabditis (C.) elegans*, *Drosophila* and mouse. Among them are PI-3 kinase (Age), TOR (Target of Rapamycin), PAK1 (RAC/CDC42-activated kinase 1) and ILK (Integrin-Linked Kinase). KO (Knock Out) of these genes extends the healthy lifespan, increases heat-endurance, and reduces brood size (fertility) of these small animals. In other words, there is a clear "trade-off" relationship between their fertility and survival. In this mini-review we focus mainly on natural or synthetic PAK1-blockers that affect both fertility and survival. Interestingly these PAK1-blockers are among anti-cancer reagents. Thus, unlike conventional anti-cancer drugs such as DNA/RNA/microtubule poisons, these PAK1-blockers could cure cancers without causing any side effects. Both melatonin and a bee product called propolis are among the "longevity-promoting" natural PAK1-blockers. Recently we and others found that even a few synthetic PAK1-blockers such as 15K, highly cell-permeable 1,2,3-triazolyl ester of an old pain killer called Ketorolac are able to boost both lifespan and heat-endurance of *C. elegans*, while they down-regulate the fertility. Here we propose a unique notion that assaying for both anti-fertility and thermo-resistance in *C.elegans* could serve as both very sensitive and time-saving in vivo screening for potent PAK1-blockers that could cure cancers and many other PAK1-dependent diseases/disorders such as AD (Alzheimer's disease) without any serious side effects.

Introduction

Major problem associated with conventional anti-cancer drugs such as DNA/RNA/ microtubule poisons is a serious un-avoidable side effect(s) such as hair-loss, suppression of immune system and loss of appetite. These side effects stem from the fact that these poisons kill mainly the fast-growing cells including the majority of malignant cells but also hair cells, bone-marrow cells and intestinal brush-border cells which divide rapidly. However, around the turn of this century, a few far more selective anti-cancer drugs have been developed. Among them is "Gleevec" that inhibits a few oncogenic Tyr-kinases such as ABL, PDGFR (Platelet-Derived Growth Factor Receptor) and KIT [1]. Gleevec cures a few specific types of cancers called CML (Chronical Myelogenous Leukemia) and GIST (Gastrointestinal Stromal Tumor) without any serious side effects. Unfortunately, however, these rare cancers represent less than 0.1 % of all human cancers. Thus, the remaining 99% of cancer patients need another set of "signalling therapeutics" that block selectively the oncogenic signal transducers such as TOR (Target of Rapamycin), PAK1 (RAC/CDC42-activated kinase 1) and ILK (Integrin-Linked Kinase), without affecting normal signal transducers.

Around two decades ago, PAK1 was recognized by us and others as an oncogenic kinase that is activated by oncogenic RAS mutants in solid tumors such as pancreatic, colon and lung cancers representing at least 30% of all human cancers. Knock-out or silencing of PAK1 gene in these RAS cancers strongly suppresses their malignant (anchorage-independent) growth, without any effect on the normal (anchorage-dependent) cell growth [2]. More recently we confirmed that PAK1-deficient mutant (RB689) of *C. elegans* lives 60% longer than the wild-type [3], clearly indicating that PAK1 is an ageing kinase which shortens the healthy lifespan of this worm. Although nobody has measured the lifespan of PAK1-deficient mice as yet, they look far healthier than the wild-type, being resistant to PAK1-dependent tumor growth [4] and LPS (Lipopolysaccharide)-induced inflammation [5].

In fact many distinct natural PAK1-blockers have been shown to extend the lifespan of small animals such as *C. elegans*, *Drosophila* and mice. Among them, melatonin was the first elixir (longevity-promoter) from bovine pineal glands [6]. Currently, melatonin is often used as a circardian sleeping pill, but it exerts many other pharmacological effects such as anti-cancer, anti-inflammatory and anti-AD (Alzheimer's disease) effects, all of which are shared with typical PAK1-blockers such as curcumin and a bee product called propolis. Propolis is a mixture of several distinct

herbal PAK1-blockers such as CAPE (Caffeic Acid Phenetyl Ester), ARC (Artepillin C) and nymphaeols from plants [7].

More recently, even a few distinct synthetic PAK1-blockers such as Minocycline (MC) were also confirmed to extend the healthy lifespan of *C. elegans* [8] or *Drosophila* [9]. Among them 15K is so far the most potent elixir that extends the lifespan of *C. elegans* even at 50 nM [8]. These PAK1-blocking elixirs share a few other common phenotypes. First of all, they down-regulate the fertility of this worm by 70-80% [3,8]. More interestingly, they boost the heat-endurance of this worm several times [8]. After prolonged heat-shock treatment (at 35°C for 8 hrs), more than 50% of the control worms die in 24 hrs, but more than 50% of the 15K-treated worms survive for more than 8 days [8]. Thus, we would easily predict that 15K and many other potent PAK1-blcokers could prevent us from a premature death (shorter lifespan) caused by the current "global warming" (heat-shock).

In this brief review we introduce several examples of "longevity-promoting" PAK1-blockers isolated from nature or chemical synthesized.

Natural PAK1-blockers

Melatonin

Melatonin is a circardian hormone released from pineal glands. Its synthesis starts normally around the sunset to induce sleep, and stops just before the sunrise every morning [6]. It is derived from another hormone called serotonin (Figure 1), and was originally identified as an anti-melanogenic compound by Prof. Aaron Lerner at Yale in 1953, and named "melatonin", meaning "anti-melanogenic serotonin derivative" [6]. However, the molecular mechanism underlying its anti-melanogenic activity remained unknown for so long until recently. It is now almost certain that melatonin suppresses the melanin synthesis by blocking the oncogenic/ageing/melanogenic kinase PAK1 [10]. Thus, it was not an entirely big surprise that melatonin extends the healthy lifespan of *Drosophila* as well as mice and rats by 20% [11]. Although melatonin is currently used as the major sleeping-pill for re-adjusting the jet-lags of trans-continental flight passengers, it could be useful for many other purposes such as cancer therapy, boosting immune response, and promoting the longevity by blocking PAK1 signaling pathway.

However, one of the major problems with melatonin is that it's high IC_{50} (around 1.3 mM against the growth of A549 cancer cell line), and we wonder if we could lower the IC_{50} by increasing its cell-permeability. However, so far an expected increase in its cell-permeability alone by replacing its acetyl moiety with basic amino acids such as ARG does not affect its anti-cancer activity, raising the possibility that its primary anti-cancer target(s) is on cell-surface rather than in the cytoplasm (Ahn MR, Kim I, Maruta H et al, unpublished observation). Interestingly, however, melatonin (1-20 μM) was recently reported to protect the LPS (Lipopolysaccharide)-induced apoptosis of HUVECs (Human Umbilical Vein Endothelial Cells) by activating AMPK (AMP-activated kinase), an anti-oncogenic kinase in the cytoplasm [12]. Thus, we are currently examining whether the ARG-linkage of melatonin potentiates its anti-LPS effect or not. Furthermore it would be of great interest to test if the ARG-linkage boosts the elixir (longevity-promoting) activity of melatonin in *C. elegans*.

Propolis

Propolis is a bee product, alcohol-extract of honey cumb, and has been used as a traditional medicine for over 4 thousand years since the ancient Egyptian era. It is among anti-biotics that are effective against both bacterial and viral infections. That is one of the major reasons why propolis was used for preparation of mummies of deceased royal families stored under pyramids for thousands years. Hypocrates, the father of medicine, in ancient Greece coined this bee product "Propolis" ("Pro" for protection and "Polis" for city or honey cumb). However, chemical components in propolis vastly differ from one to another depending on regions where propolis is harvested. For example, propolis from Europe, Far-East, and Oceania is rich in CAPE (Caffeic Acid Phenethyl Ester) and CA (Caffeic Acid), whereas Brazillian green propolis is rich in ARC (Artepillin C), and those from Asia-Pacific subtropical areas such as Okinawa, Taiwan and Hawaii are rich in nymphaeols [7,13]. Nevertheless all propolis products share a very unique common biological property: blocking PAK1, the major oncogenic/ageing/melanogenic kinase, and causing basically no side effect [7,13].

In late 1980s, CAPE-based propolis was found to inhibit the growth of cancer cells, but not the normal cell growth [14]. Since then propolis has been used as an alternative cancer therapeutic for treatment of cancer patients who fail to respond to the conventional anti-cancer drugs (DNA/RNA/microtubule poisons). More recently propolis was shown to extend the healthy lifespan and boost the heat-endurance of *C. elegans* [3,15, 16]. One of the major problems associated with propolis is poor water-solubility (low bioavail-ability) and poor cell-permeability as well. In an attempt to overcome these problems, we have converted the COOH-bearing ARC and CA to 1,2,3-triazolyl ester via Click Chemistry (CC), boosting their cell-permeability and anti-cancer activity by 100-400 folds without loss of their water-solubility [17].

Curcumin

Curcumin is the major PAK1-blocking ingredient in Turmeric roots and directly inhibits PAK1 with IC_{50} around 16 μM [15]. It is chemically similar to CAPE, and inhibits the growth of a variety of cancer cells such as A549 with IC_{50} around 23 μM [15]. Curcumin also has been shown to extend the lifespan of *C. elehgans* [18]. The major problem/setback for clinical application of curcumin is its poor bio-availability (water-insolubility). A decade ago, an oncologist

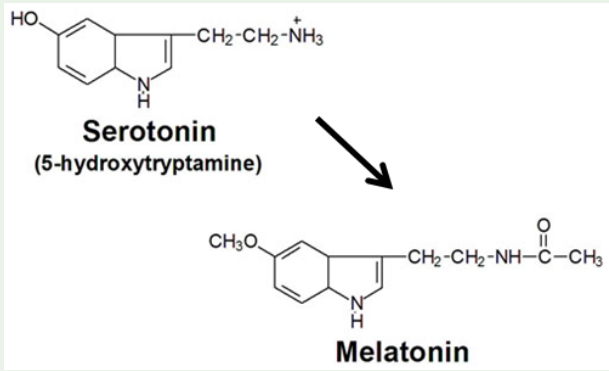

Figure 1: Biosynthesis of Melatonin.

group led by Razelle Kurzrock at MD Anderson Cancer Center in Texas successfully potentiated the bioavailability of curcumin by liposomes for therapy of pancreatic/colon cancers [6], and according to their 2008 clinical trial report, this "liposome" recipe appears to work in clinical trials (phase II) for some advanced pancreatic cancer patients [6].

Triptolide

Tripterygium wilfordii, sometimes called thunder god vine, is a vine used in traditional Chinese medicine. An extract of its leaves or stalks has been used for controlling rheumatoid arthritis and other inflammatory diseases. It has been used for birth control for men as well, as it inhibits the growth of sperms. However, in 1972, its major anti-cancer ingredient was identified by Bryan and his colleagues as a di-terpenoid called Triptolide (TPE), (Figure 2), and its activity against the growth of pancreatic cancer and AD (Alzheimer's disease) has been revealed [19]. However, the molecular mechanism underlying its anti-cancer activity remained unknown till recently. In 2009, a Chinese group found that Triptolide inactivates PAK1 by inhibiting both RAC and JAK2 [20]. TPE inhibits the growth of pancreatic and colon cancer cells with IC_{50} around 30 nM, and in vivo inhibits the growth of human pancreatic and colon cancer xenografts in mice with 0.3 mg/kg [20]. In 2017, we found that it extends the lifespan of *C. elegans* by 20% and boosts the heat-endurance at 140 μM, proving that TPE causes no serious side effect [21]. However, since it is water-insoluble, it is not suitable for clinical application. Thus, in 2015, a group at Minnesota University synthesized a water-soluble phosphoryl derivative of Triptolide called "Minnelide" [22], and its clinical trial (phase II) for pancreatic cancer has been initiated.

Glaucarubinone

Glaucarubinone is a triterpenoid/quassinoid derived from a bitter tree (*Simarou-baceae* family) in Amazon forest. The extract of this tree bark has been used as a traditional medicine by local Amazon people for treatment of a variety of diseases including malaria infection which is now proven to be PAK1-dependent. Around 1981, its major active ingredient was identified as glaucarubinone, and rather surprisingly, it was found to show a potent anti-cancer activity [23]. However, the molecular mechanism underlying its anti-cancer activity remained to be clarified until recently. In 2009, John Beutler's group at NCI-Frederick found the first clue: this compound inhibits the function of an oncogenic transcription factor called AP-1 in cancer cells with IC_{50} around 20 nM [24]. Since AP-1 is downstream of PAK1, we started suspecting that it might be a PAK1-blocker. In collaboration with his team, we confirmed that glaucarubinone indeed blocks PAK1 in cell culture, and another group led by Hong He at Melbourne University Hospital found that it blocks both PAK1 and PAK4 in vivo, inhibiting the growth of human pancreatic and colon cancer xenograft in mice at 1-2 mg/kg, i.p. twice a week [25]. To the best of our knowledge, this compound is the most potent anti-cancer agent among herbal PAK1-blockers. Most interestingly, in 2011, a group at Jena University in Germany found that this compound at 1-10 nM extends the lifespan of *C. elegans*, suggesting that it would not cause any side effect during cancer therapy [26].

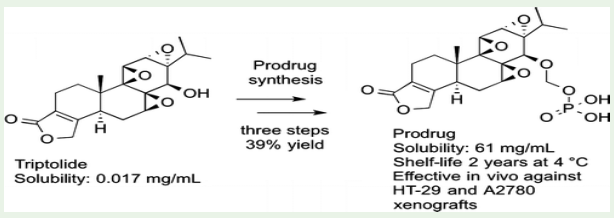

Figure 2: Triptolide and its "pro-drug" (Minnelide).

Daumone

Daumone is a dauer-inducing glucosylated pheromone (Figure 3) produced by *C. elegans* [27]. It was originally found to be a potent anti-cancer agent by a Korean group at Yonsei University led by Mankil Jung in 2005 [27]. It can be chemically synthesized as well. In 2009, this team managed to synthesize a very potent anti-cancer derivative from Daumone with IC_{50} around 20 nM [28]. Later (in 2014), Daumone (2 mg/kg daily) was found by the same team to extend the healthy lifespan of mice by 50% [29]. Since Daumone or its derivatives inhibit strongly both PAK1-dependent inflammatory diseases and angiogenesis in ovo (CAM assay) as well [28], it is almost certain that Daumone also blocks PAK1 somehow, and we are planning to confirm this notion. More interestingly, Daumone bears a COOH moiety, and if it is esterized with the water-soluble triazolyl alcohol via Click Chemistry (CC), its anti-cancer activity could be boosted with a robust increase in its cell-permeability, as described earlier with a few other COOH-bearing PAK1-blockers such as ARC and CA [18].

Synthetic PAK-blockers

1,2,3-Triazolyl ester of ketorolac (15K)

Ketorolac, an old pain-killer, is a racemic mixture of R-form and S-form. R-form inhibits RAC, blocking PAK1 [30], while S-form directly inhibits COX-2 which is involved in the production of prostaglandin [30]. The growth of A549 cancer cells requires both PAK1 and its effector COX-2 [31]. However, just like Daumone, ARC and CA, Ketorolac is among COOH-bearing PAK1-blockers whose cell-permeability is rather poor. Thus, we recently linked its COOH moiety to the water-soluble 1,2,3-triazolyl alcohol via CC, making a highly cell-permeabble ester called "15K" (Figure 4). Both anti-cancer and anti-PAK1 activities of Ketorolac are boosted over 500 times by the "CC"-based esterization. In addition, its anti-COX-2 activity is boosted 20 fold, most likely due to the anti-COX-2 activity of 1,2,3-Triazolyl ring per se [31].

In ovo (CAM assay in fertilized chicken eggs), "15K" was shown to inhibit the angiogenesis with IC_{50} = 1 nmol/egg [32]. Furthermore, "15K" (50 nM) extends the healthy lifespan of *C. elegans* by 15-30%, and boosts the heat-endurance by 10-fold even at 10 nM [8]. To the best of our knowledge, 15K is the most potent synthetic "elixir" (longevity-promoter).

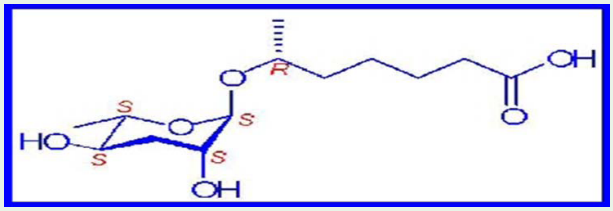

Figure 3: Daumone.

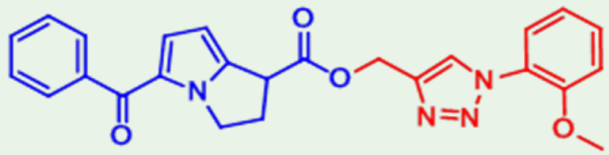

Figure 4: 1,2,3-triazolyl ester of ketorolac (15K).

Aspirin

The most popular Bayer product called Aspirin (acetylsalicylic acid) is the oldest semi-synthetic pain-killer originally derived from an herbal medicine called salicylic acid (SA). Interestingly, however, Aspirin was found to inhibit both inflammation and cancer growth with IC_{50} around 3 mM. A few years ago, a Chinese group reported that Aspirin extends the lifespan of *C. elegans* and boosts its heat-endurance [33]. Due to its COOH-moiety, however, its cell-permeability is rather poor. Thus, several years ago, via CC, Aspirin was also converted to 1,2 3-triazolyl ester. IC_{50} of this ester against the growth of A549 cancer cells is around 0.1 mM, 30 times lower than that of Aspirin.

Metformin

Metformin is an old synthetic anti-diabetic/anti-obesity compound. The major target of metformin is the anti-oncogenic LKB1-AMPK (AMP-activated kinase) cascade, and metformin activates AMPK with ED_{50} around 2 mM [34]. Interestingly, however, almost all AMPK activators inhibit PAK1 by activating LKB1 which in turn inactivates PAK1 [7]. Thus, it is most likely that Metformin is also among PAK1-blockers. A few years ago Metformin was shown to extend the healthy lifespan of *C. elegans* at 50 mM [35].

Minocycline

Minocycline (MC), (Figure 5) is an old semi-synthetic derivative of tetracycline developed as an improved antibiotic by Lederle in 1961 and came into commercial use in 1971. However, over decades, in addition to the anti-bacterial activity, MC has shown a variety of other pharmacological activities including an anti-cancer activity that inhibits the growth of A549 cancer cells with IC_{50} ranging 5-10 μM [36], as well as the PAK1-dependent blood-coagulation with apparent anti-MLKs (RAC/CDC42-activated kinases) activity [37]. Recently MC at 50 μM was found to extend the lifespan of *Drosophila* by 25% [38]. More recently, MC was reported to prevent cerebral malaria in mice [39], clearly indicating that MC passes through BBB (Blood

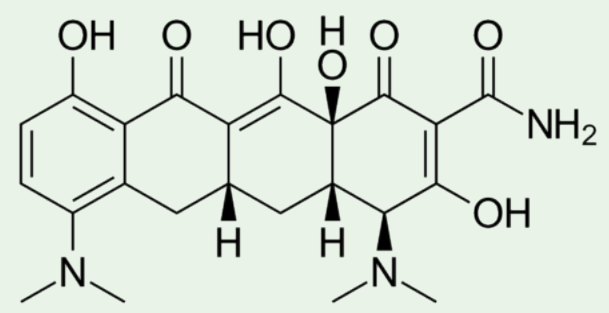

Figure 5: Minocycline.

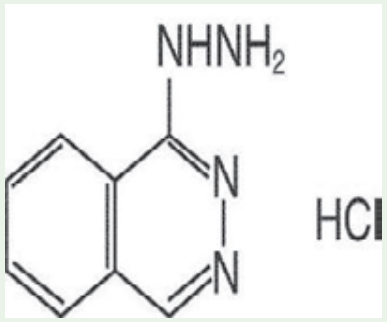

Figure 6: Hydralazine-HCl.

Brain Barrier). In support of this notion, MC has been shown to be effective clinically in promoting sleep-associated memory processing [40] and for treatment of brain tumors such as NF1-deficient MPNST [41]. Regarding the molecular mechanism underlying its anti-cancer, anti-malaria and elixir (longevity-promoting) activities, it is most likely that MC directly inhibits both PAK1 and MLKs, as does CEP-1347 [42].

Hydralazine

Hydralazine (Figure 6) is a medication used to treat high blood pressure and heart failure. It was originally developed by Chiba-Geigy for Malaria treatment and patented in 1949. Both malaria and hipertesion are among PAK1-dependent diseases, it is most likely that hydralazine is among synthetic PAK1-blockers. Thus, it is not a surprize that hydralazine was recently found to extend the lifespan of *C. elegans* and induces stress-resistance as well [43].

Conclusion

The combination of both reduction of fertility and increase in heat-endurance of *C. elegans* could serve as the most sensitive (and time-saving) criteria (or indicators) for us to screen in vivo a variety of PAK1-blocking anti-cancer drugs/elixirs such as melatonin, propolis and 15K that do not cause any serious side effects. Furthermore, while PAK1-deficient mice are highly resistant to LPS-induced inflammation [5], CD300f-deficient mice are highly senstive to LPS-induced inflammation/atopy [44], strongly suggesting the possibility that CD300f, a ceramide receptor, is a natural PAK1-blocking tumor suppressor/elixir. Thus, a LPS-induced skin reaction/atopy at ears of CD300f-deficient mice could also serve as a rapid and inexpensive in vivo screening system for PAK1-blockers.

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