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# feature



## Chemical evolution for taming the 'pathogenic kinase' PAK1

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To celebrate the 25th anniversary of the cloning of the first mammalian p21-activated kinases (PAKs) (RAC/CDC42-activated kinases) by Ed Manser, the first international PAK symposium was held in NYC in October 2019. Among six distinct PAKs in mammals, PAK1 is the major 'pathogenic kinase', the abnormal activation of which is responsible for a wide variety of diseases and disorders including cancers, ageing processes and infectious and inflammatory diseases such as pandemic coronaviral infection. Recently, for a clinical application, a few potent (highly cell-permeable and water-soluble) PAK1 blockers have been developed from natural or synthetic PAK1 blockers (triptolide, vitamin D3 and ketorolac) via a series of 'chemical evolutions' that boost pharmacological activities >500 times.

### Amoeba PAK (myosin I heavy chain kinase)

More than four decades ago, an NIH team discovered a peculiar kinase in a soil amoeba that phosphorylated the heavy chain of an unusual myosin: a single-headed myosin (myosin I), and this phosphorylation led to a robust activation of myosin I ATPase by actin fiber (F-actin) [1]. Interestingly, myosin I is essential for amoeboid movement including phagocytosis and cell migration but not for cell division (cytokinesis), which depends on another myosin: double-headed myosin (myosin II). Curiously, the amoeba myosin I heavy-chain kinase phosphorylates the regulatory light chain of smooth muscle myosin II from mammals, leading to a robust activation of this muscle myosin II ATPase by F-actin. Therefore, if such a kinase should exist in mammals as well, the mammalian counterpart was anticipated to raise the blood pressure by triggering smooth muscle contraction along blood vessel walls.

### Mammalian PAK

Eventually, 17 years later, according to an early 1994 issue of *Nature* [2], a similar kinase was discovered in the mammalian brain by a British scientist (Dr Ed Manser) in Singapore – and named p21-activated kinase (PAK)1 (RAC/CDC42-activated kinase 1). Because the G proteins called RAC and CDC42 were known to be activated by the oncogenic G protein RAS, which is responsible for >30% of malignant transformation of human cells (such as pancreatic, colon and lung cancers), we wondered whether PAK1 might be responsible for RAS-induced malignant transformation. Soon, Ed Manser's team discovered another protein called PIX, which is essential for the activation of PAK1, and found that the PIX–PAK1 interaction is essential for a phenomenon called 'membrane ruffling' of cancer cells [3,4]. The SH3 domain of PIX binds to the Pro-rich domain of PAK1 called PAK18, consisting of 18 amino acids [4]. Because the actomyosin-based membrane ruffling is

closely linked to RAS-induced malignant transformation, a team at Ludwig Institute for Cancer Research (Melbourne Branch) synthesized a highly cell-permeable peptide called WR–PAK18, in which the cell-permeable (Trp- and Arg-rich) peptide vector WR is linked to PAK18, and they confirmed that WR–PAK18 inhibits the RAS-induced malignant transformation with an IC<sub>50</sub> below 5 μM by blocking the PIX–PAK1 interaction in cells [5]. This peptide was the very first PAK1 blocker that suppressed the growth of cancer cells selectively, without affecting normal cell growth.

### PAK1-dependent diseases and disorders

Since then, by means of WR/TAT-PAK18 and many other PAK1 blockers, it has been revealed that abnormal activation of PAK1 is responsible not only for cancers but also for so many other diseases and disorders such as hypertension, diabetes (type 2), infectious and inflammatory diseases including coronavirus pandemic,

neuronal diseases such Alzheimer's disease (PD), Parkinson's disease (PD), epilepsy, schizophrenia, depression, autism, immune-suppression, osteoporosis, hair loss and hyper-pigmentation [6,7]. Furthermore, it was found that a PAK1-deficient mutant of *Caenorhabditis elegans* lives 60% longer than the wild-type [6], proving that even a normal level of PAK1 is sufficient to shorten the healthy lifespan. Thus, in theory, PAK1 blockers could promote longevity as well. In fact, several natural PAK1 blockers such as propolis and melatonin significantly extend the healthy lifespan of small animals such as *C. elegans* and mice [6,7]. Undoubtedly, therefore, the potential market value of PAK1 blockers could be huge in the pharmaceutical and cosmetics industries. Thus, celebrating the 25th anniversary of the mammalian pathogenic kinase (PAK1) cloning, the first international PAK symposium (12 October 2019) entitled 'Pathogenic Roles of PAK1: Anti-PAK1 Therapy Promoting the Longevity' was held in New York City (<https://www.somatopublications.com/pathogenic-roles-of-pak1-including-oncogenesis-and-ageing.pdf>).

Among six distinct members of the PAK family in mammals, only PAK1 and PAK4 turned out to be pathogenic (in particular oncogenic). However, because CDC42-activated kinase 4 (PAK4) is essential for embryogenesis [6], developing PAK1 blockers has been the focus for clinical application. Although a bunch of reviews on PAK1 have been published during the past several years, most of them concern only PAK1 inhibitors that are useful only as laboratory reagents, and they are basically useless for clinical application, mainly owing to either poor water solubility or poor cell permeability.

### Natural PAK1 blockers

Instead, we have focused on identifying or developing a series of clinically useful PAK1 blockers (natural or synthetic) and their chemical potentiation for the past two decades [7]. One of the natural PAK1 blockers available on the market turned out to be a bee product (alcohol-extract of beehives) called propolis. Around 1988, caffeic acid phenethyl ester (CAPE)-based propolis was found, by a team at Columbia University, to kill cancer cells selectively without any effect on normal cell growth [8]. However, the molecular mechanism underlying propolis therapy of cancers remained unknown until 2005 when CAPE or caffeic acid (CA) in propolis was revealed to downregulate RAC, just upstream of PAK1 [9]. Interestingly, although the major anticancer ingredients in propolis vastly differ from one product to another, depending on the major plant sources from which bees prepare hives, all propolis

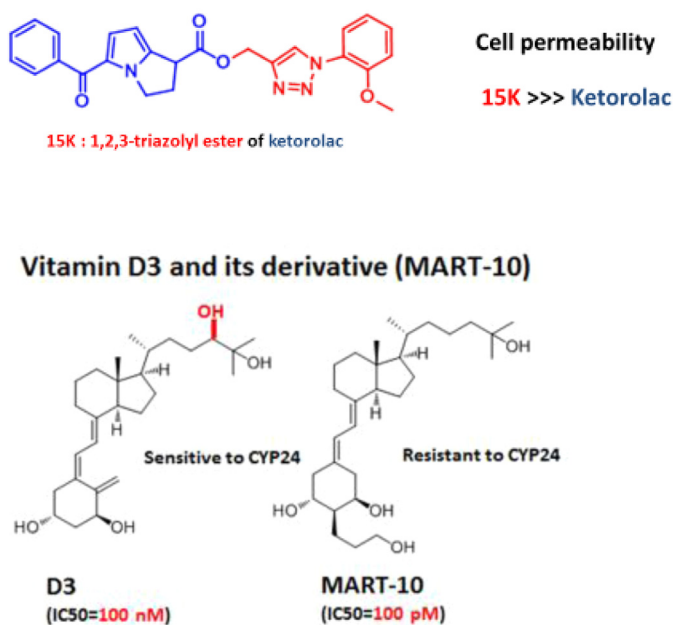
products contain PAK1 blockers such as CAPE, artemillin C (ARC) and nymphaeols, so far without any exception [10]. Moreover, unlike conventional anticancer drugs (chemotherapeutics), which cause several serious side effects such as immune suppression and hair loss, propolis caused no side effects and even promotes the immune system, hair growth and longevity [11,12].

### Highly cell-permeable synthetic PAK1 blockers

Either water insolubility or poor cell permeability (therefore low bioavailability) of propolis has been the major problem limiting worldwide clinical application. For example, ARC and CA bear the COOH moiety which blocks their free penetration through negatively charged plasma membranes of target cells, whereas CAPE is poorly water-soluble. Thus, a few years ago, our Melbourne team decided to potentiate these PAK1 blockers of propolis origin by a unique esterization with a water-soluble 1,2,3-triazolyl alcohol via click chemistry (CC), which was developed by Barry Sharpless (2001 Nobel Laureate) and his team [13]. Mainly owing to a robust increase in their cell permeability, 1,2,3-triazolyl ester of ARC (15A) and of CA (15C) are 100- and 400-times more potent in anticancer and anti-PAK1 activities than ARC and CA, respectively, with  $IC_{50}$ s  $\sim$ 200 nM [14]. Eventually from an old pain killer (ketorolac), we developed a far more

effective PAK1 blocker called 15 K via CC [15] (Fig. 1a).

Ketorolac is a synthetic racemic compound and its S-form has been long known to inhibit cyclooxygenase (COX)-2 directly, COX-2 is responsible for synthesis of prostaglandin (a pain source). Interestingly, a few years ago its R-form was found by a team at University of New Mexico to downregulate RAC, thereby blocking PAK1 with an  $IC_{50} \sim 13 \mu\text{M}$  [16]. However, ketorolac bears a COOH moiety that hampers its free penetration through the negatively charged plasma membranes of target cells; and tends to cause an ulcer as well if used regularly. Thus, we decided to boost its cell permeability by esterization with 1,2,3-triazolyl alcohol via CC as well [15]. This esterization led to a great leap (>500 times) in the anti-PAK1 and anticancer properties of ketorolac [15]. The  $IC_{50}$  of 15 K is in the range 5–24 nM against different cancer cell lines, and it increases the heat resistance of *C. elegans* by nine times even at 10 nM  $\sim 35^\circ\text{C}$  (nonpermissive temperature), inducing a heat shock gene called HSP16.2 via the longevity transcription factor FOXO, and concomitantly extends the healthy lifespan of this worm by 15–30%  $\sim 20^\circ\text{C}$  (permissive temperature) [17]. Facing the current climate crisis namely 'global warming or extreme heat' (e.g., frequent summer bushfires in California and the southern half of Australia), 15 K and other PAK1 blockers could provide us with a practical alternative (chemical



**FIGURE 1**

(a) 15 K: highly cell-permeable ester of ketorolac. (b) Vitamin D3 and its [cytochrome P450 (CYP)24-resistant] derivative MART-10. Hydroxylation of D3 at position 24 (highlighted in red) by human CYP24 inactivates D3.

evolution) of the genetic evolution (knockout of PAK1 gene) for our survival of the extreme heat, which might eventually lead to extinction of the whole animal kingdom from the surface of this planet (none of the plant kingdom carries the pathogenic PAK1 gene). If we understand this correctly, once the animal kingdom (in particular the over-populated *Homo sapiens*) perishes, the earth would be cooled down, back to another ice age.

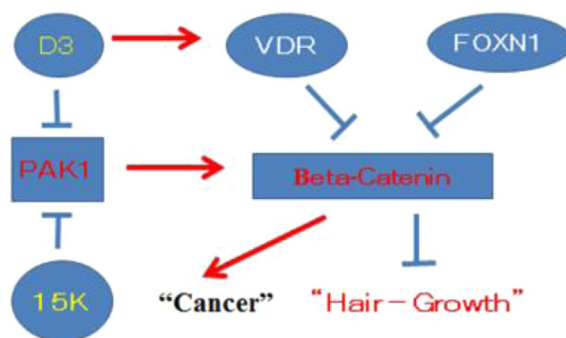
Notably, 90% of human pancreatic cancers are resistant to chemotherapies such as gemcitabine (GEM), and their chemoresistance is mainly the result of chemo-induced abnormal activation of PAK1 [18]. Remarkably, 15 K suppresses growth and metastasis of GEM-resistant human pancreatic cancer xenografts in mice with an  $IC_{50}$  below 0.1 mg/kg/day, causing no side effect in mice even with doses of 5 mg/kg/day [19].

### Vitamin D3 and its potent derivative MART-10

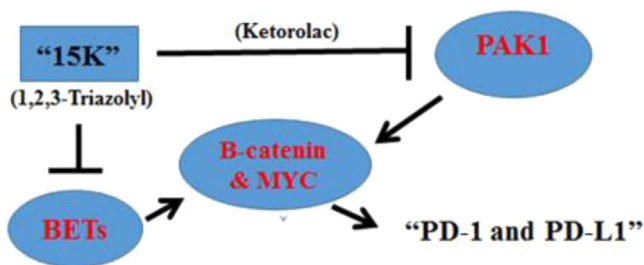
The best-known physiological role of vitamin D3 is calcemic action promoting calcium absorption into bone tissues. However, back in 1987, one of its new roles was discovered by an Australian team: suppressing the growth of colon cancer xenografts in mice even with 5  $\mu$ g/kg/day on low-calcium diet taming its calcemic effect [20]; but not melanomas which lack D3 receptor (VDR), strongly suggesting that the anticancer activity of D3 is also through VDR, a nuclear transcription factor. However, a D3 metabolite, which is hydroxylated at position 24 (Fig. 1b) by an enzyme called CYP24, was ineffective, mainly owing to its failure in activating VDR.

Two decades later, a team at Teikyo University in Tokyo developed a series of vitamin D3 derivatives. Among them is MART-10 (Fig. 1b) which is 100-times more-resistant to CYP24 [21]. To their great surprise, the anticancer activity of MART-10 ( $IC_{50}$  ~100 pM) was found to be 1000-times more potent than D3 ( $IC_{50}$  ~100 nM), although it is poorly calcemic [21]. Excitingly, D3 was recently found by a German team to downregulate RAC, thereby blocking the PAK1/LIM kinase/cofilin pathway and depolymerizing actin filaments [22]. In other words, there are at least two distinct D3-induced anticancer signaling pathways: one activating VDR and the other inactivating RAC/PAK1, both eventually downregulating an oncogenic transcription factor called  $\beta$ -catenin (Fig. 2a). Furthermore, it was revealed that MART-10 also depolymerizes actin filaments in cancer cells, strongly suggesting that MART-10 is also a potent PAK1

### D3-induced “anti-cancer/anti-alopecia” signalings



### 15K: an immune (check-point) therapeutic of cancers suppressing both PD-1 and PD-L1 via PAK1 and BETs signalings



**FIGURE 2**

(a) D3-induced anticancer/anti-alopecia signaling pathways. (b) 15 K: a potent immune (checkpoint) cancer therapeutic suppressing PD-1 and PD-L1 via p21-activated kinase (PAK1) and bromodomain and ex-terminal domain proteins (BETs) signaling. 15 K blocks PAK1 via ketorolac and BETs through the 1,2,3-triazolyl ring, suppresses their common downstream targets ( $\beta$ -catenin and MYC) and eventually PD-1 and PD-L1 expression.

blocker [23]. D3 was known to extend the healthy lifespan of *C. elegans* [24], and MART-10 suppresses growth and metastasis of GEM-resistant human pancreatic cancer xenografts in mice with an  $IC_{50}$  below 0.1 mg/kg/day [25]. Thus, 15 K and MART-10 (made in Japan) are among the most potent synthetic PAK1 blockers, and both of us are very keen to initiate their clinical trials for GEM-resistant pancreatic cancers as soon as possible with a generous financial support and/or backup from one of the keen pharmaceutical giants.

A third potent synthetic PAK1 blocker called minnelide, a water-soluble phosphatase-sensitive prodrug (phosphorylated form) of triptolide, was synthesized by a team at University of Minnesota led by Gunda Georg, a German organic chemist. This also inhibits RAC with an  $IC_{50}$  ~30 nM [26] and is currently in clinical trials (Phase II) for cancers by Minneamrita Therapeutics in the USA. Amazingly, the phosphorylation of triptolide alone boosts its water solubility >3000 times, thereby its bioavailability as well [27].

### Pathogenic roles of PAK1 in immune system and hair growth

Back in 1962, upon a spontaneous genetic mutation, a hair-less and thymus-deficient mouse strain: the nude (nu/nu) mouse, was isolated by Norman Grist in Scotland [28]. The lifespan of nu/nu mice is only 6 months, one-fifth compared with the wild-type, mainly because of no immunological defense against a wide variety of pathogenic bacteria and viruses. However, under sterile conditions and controlled temperatures, they could live as long as the wild type. Because nu/nu mice do not reject either allografts or xenografts, they have often been used to test the anticancer effects of new drugs against human cancer xenografts, before their clinical trials. Around 2000, deficiency of a gene called *FOXN1* was found to be solely responsible for their phenotypes (i.e., hair-less with thymic deficiency) [28,29]. However, the signaling pathway(s) underlying *FOXN1*-dependent hair-growth and thymus development remained unknown until recently.



Finally in 2018, a Chinese group solved this mystery. Downregulation of *FOXN1* in human lung cancer cells (A549) activates the  $\beta$ -catenin gene, just downstream of PAK1, promoting the growth of cancer cells [30], clearly indicating that *FOXN1* is a tumor suppressor gene that blocks the oncogenic PAK1/ $\beta$ -catenin pathway. This finding is not entirely a surprise, because in 2002 a few potent D3 analogs developed by Milan Uskokovic's team at Roche promoted hair-growth in the *nu/nu* mice even with the daily dose below 50 ng/kg [31]; and a few other natural PAK1 blockers such propolis, melatonin and cucurbitacins also promoted hair growth in cell culture or *in vivo* [7,32,33]. It is also worth noting that VDR-null mice are also hair-less, and D3 could restore hair-growth in VDR-null mice [34], probably by inactivating RAC/PAK1/ $\beta$ -catenin signaling [35] (Fig. 2a). Interestingly, downregulation of PAK1/ $\beta$ -catenin signaling in mice also boosts their immune system [36].

Although a PAK1-KO mutant of *C. elegans* lives 60% longer than the wild type [6], so far nobody has been motivated to examine the healthy lifespan of PAK1-KO mice as yet, partly because even the wild-type lives as long as 3 years (>75-times longer than *C. elegans*). Nevertheless, it is expected that PAK1-deficient mice live significantly longer than the wild-type, mainly because mice treated with either melatonin or rapamycin, in which PAK1 or TOR (an oncogenic kinase downstream of PAK1) is suppressed, are far more resistant to pathogens and stresses such as coldness and UV light in the wildness, unlike the 'nude' (*FOXN1*-deficient) mice, and indeed live significantly longer than the control wild mice [6,7]. In other words, phenotypes of PAK1-deficient mice must be precisely opposite to those of the fragile short-lived *nu/nu* mice.

### The ED therapeutic Viagra® is also a PAK1 blocker

More than two decades ago, Pfizer marketed a new antihypertension drug called sildenafil mainly for the therapy of erectile dysfunction (ED) under the brand name Viagra®. It is a cyclic GMP analog that inhibits selectively the cyclic GMP phosphodiesterase (PDase 5) with an  $IC_{50}$  below 5 nM [37]. It is well known that 1 in 6 males suffer from ED worldwide. However, it was recently revealed that Viagra® suppresses the PAK1-dependent expression of PD-L1 in human lung cancer cell lines such as A549 by downregulating histone deacetylases (HDACs) with the  $IC_{50}$  ~100 nM [38], which eventually blocks PAK1 [6] (Fig. 3). Thus, a combination of Viagra® and anti-PD-L1 monoclonal antibodies (checkpoint therapeutics) is currently in clinical trials

## “Viagra” blocks PAK1 by down-regulating HDACs

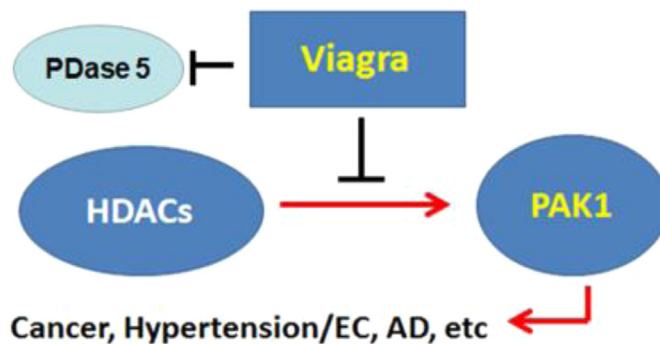


FIGURE 3

Viagra® blocks p21-activated kinase (PAK1) by downregulating histone deacetylases (HDACs).

for cancers. Furthermore, back in 2009, a group at Columbia University reported that Viagra® (3 mg/kg i.p.) effectively restores memory loss in an Alzheimer's disease (AD) model of mice [39]. Because AD is also among the PAK1-dependent diseases, these findings strongly suggest that Viagra® is a potent PAK1 blocker already on the market. Interestingly, unlike a more potent HDAC inhibitor FK228 (brand name Istodax®), which unfortunately fails to pass through the blood-brain barrier (BBB), Viagra® is able to cross the BBB effectively, and therefore could be potentially useful for therapy of a wide variety of PAK1-dependent neuronal diseases. It is also predicted that Viagra® could contribute to the extension (improvement) of our healthy lifespan, in particular of males, if it is properly used.

### Suppression of PD-1 and PD-L1 by 15 K via the PAK1 and BET signaling pathways

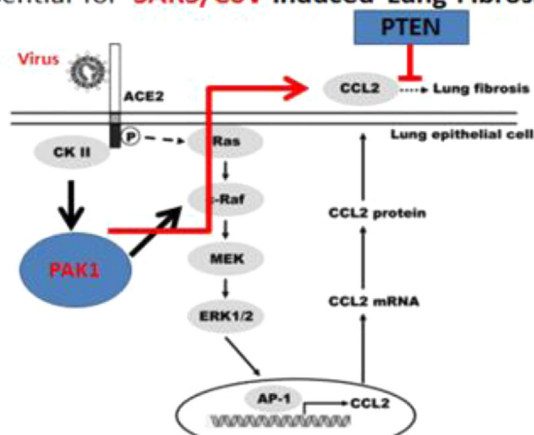
According to a 2018 Nobel Laureate, Tasuku Honjo at Kyoto University, the interaction of a ligand called PD-L1 in cancer cells with its receptor called PD-1 in T cells destroys the anti-cancer T cells responsible for so-called immunosurveillance of cancers. Thus, monoclonal antibodies against PD-L1, PD-1 or both could be useful for cancer therapy – so-called checkpoint immunotherapy. Interestingly, several PAK1 blockers such as triptolide, curcumin, resveratrol and melatonin have been shown to suppress the expression of PD-L1, strongly suggesting that PD-L1 expression might depend on PAK1 [40]. In fact, recently this notion or hypothesis was proven genetically in mice and pancreatic cancer cells by a team at Melbourne University [41]. Thus, it is possible that the potent PAK1 blocker 15 K also suppresses PD-L1 expression.

In this context, it is worth noting that the new role of bromodomain and ex-terminal domain proteins (BETs) was recently identified. BETs are responsible for expression of PD-1 and PD-L1 via the  $\beta$ -catenin/MYC pathway [42]. Furthermore, a few years ago, a series of 1,2,3-triazol compounds were found to inhibit BETs [43]. Because 15 K is also among 1,2,3-triazol compounds, we are planning to test whether 15 K suppresses PD-1 and PD-L1 by blocking not only PAK1 but also BETs, both of which are essential for the activation of the oncogenic  $\beta$ -catenin/MYC pathway (Fig. 2b). If this working hypothesis is proven, it is most likely that 15 K could be a more effective (safer and less expensive as well) therapeutic than the Nobel-prize-winning monoclonal-based (checkpoint) therapeutics that have never worked for the treatment of brain tumors and pancreatic cancers.

### PAK1 blockers for alternative therapeutics of coronavirus infection

Since the end of 2019, deadly coronavirus infection started to spread rapidly worldwide from Wuhan in China, at the time of writing >60 000 people were infected, and the death toll had reached >1500 people. Traditionally, a specific vaccine would be the way forward to combat viral diseases. However, it would take at least 6 months (and realistically 18 months) to prepare a vaccine (according to WHO). Until that time, it was feared the majority of coronavirus victims would perish. However, very recently, a team at University Colorado found that a tumor-suppressing phosphatase called phosphatase and tensin homolog (PTEN), which inactivates PAK1, could suppress the coronavirus-caused CCL2-dependent fibrosis of lungs [44]. Furthermore, a

### ACE2-CKII/RAS-PAK1-RAF—CCL2 signaling pathway essential for SARS/CoV-induced Lung Fibrosis



**FIGURE 4**

Phosphatase and tensin homolog (PTEN), a p21-activated kinase (PAK1) blocker, interferes with coronavirus-induced PAK1-dependent signaling pathway leading to lung fibrosis. Adapted, with permission, from [44,45].

decade ago, a group at Taipei found that expression of CCL2 in lung cells depends on the oncogenic signaling pathway including transmembrane viral receptor called angiotensin-converting enzyme 2 (ACE-2), kinases CK2, PAK1, RAF and the transcription factor AP-1 [45] (Fig. 4).

Interestingly, a few years after the 2003 SARS outbreak, a CDCP team at Atlanta reported that an old antimalaria drug called chloroquine suppresses the SARS/coronaviral infection in cell culture with an  $IC_{50} \sim 1 \mu M$  [46]. Malaria infection requires PAK1 in host cells [6], and chloroquine upregulates p21 (a CDK inhibitor) whose expression is suppressed by PAK1 [6,47]. Its anticoronaviral effect was recently confirmed *in vitro* by a team at the Chinese Academy of Sciences as well (<https://www.nature.com/articles/s41422-020-0282-0>). Thus, these encouraging findings altogether strongly suggest, albeit not yet proven clinically, that PAK1 blockers such as propolis and chloroquine currently available on the market could be potentially useful for treating or easing the pandemic coronavirus infection, until its vaccine becomes available.

#### Concluding remarks

In the past (over a decade ago), development of anti-PAK1 drugs conducted mainly by pharmaceutical giants focused on PAK1 specificity. However, in a common sense it would be very difficult to imagine that a given compound inhibits only PAK1, and not any other kinases or enzymes. Instead, here we have emphasized a chemical evolution of pre-existing natural or

synthetic compounds known to inhibit PAK1 directly or upstream enzymes; but without any side effect *in vivo* (in small animals such as *C. elegans* and mice). This approach could boost either their cell permeability, water solubility or metabolism resistance. In a sense this 'wise' (reasonably flexible) approach simply follows the million-plus years of history regarding our own genetic and cultural evolution from primates that Charles Darwin revealed >150 years ago. Lastly it should be highlighted that the major purpose of PAK1 blockers for clinical application is to reduce the abnormally activated PAK1 to the 'normal' level (instead of 'null' level) in patients suffering from PAK1-dependent diseases. Thus, it is very unlikely that such a cautious measure or approach would cause any serious side effect.

#### Conflicts of interest

Neither author has any conflicts of interest to declare.

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H.M. wrote a large part of this Feature and A.K. was in charge of the MART-10 section.

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