Comment

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Rapamycin *vs* TORin-1 or Gleevec *vs* Nilotinib: Simple chemical evolution that converts PAK1-blockers to TOR-blockers or *vice versa*?

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SUMMARY Both PAK1 (RAC/CDC42-activating kinase 1) and TOR (Target of Rapamycin) are among the major oncogenic/ageing kinases. However, they play the opposite role in our immune system, namely immune system is suppressed by PAK1, while it requires TOR. Thus, PAK1-blockers, would be more effective for therapy of cancers, than TOR-blockers. Since 2015 when we discovered genetically that PDGF-induced melanogenesis depends on "PAK1", we are able to screening a series of PAK1-blockers as melanogenesis-inhibitors which could eventually promote longevity. Interestingly, rapamycin, the first TOR-inhibitor, promotes melanogenesis, clearly indicating that TOR suppresses melanogenesis. However, a new TOR-inhibitor called TORin-1 no longer suppresses immune system, and blocks melanogenesis in cell culture. These observations strongly indicate that TORin-1 acts as PAK1-blockers, instead of TOR-blockers, *in vivo*. Thus, it is most likely that melanogenesis in cell culture could enable us to discriminate PAK1-blockers from TOR-blockers.

Keywords PAK1, TOR, melanogenesis, immune system, rapamycin, TORin-1, Gleevec, nilotinib

1. Introduction

In mid-1994, a new Ser/Thr-kinase called "TOR" was identified in both yeast and mammals, by four groups independently, among the direct "targets" of rapamycin, an antibiotic found in Easter Island around 1970s (*1-4*). Around the very beginning of 1994, the first mammalian Ser/Thr-kinase coined "p21-activated kinase 1" (PAK1) was cloned by Ed Manser's team at Singapore National University (*5*), which is activated by two small G proteins called RAC and CDC42 (p21). Very interestingly, PAK1 is highly homologous to an amoeba kinase called "myosin I heavy chain kinase" (MIHCK), which we discovered in a soil amoeba in 1977 (*6,7*). MIHCK is essential for F-actin-dependent activation of a single headed myosin (myosin I) ATPase. This amoeba kinase phosphorylates the regulatory light chain of smooth muscle myosin II (double-headed) ATPase as well, which becomes F-actin-activatable upon the phosphorylation (*8*). This finding suggests an interesting possibility that PAK1 might be involved in hypertension. Around the turn of this century, both PAK1 and TOR were first identified among major "oncogenic" kinases (*9-12*). Later both kinases were recognized as the major "ageing" kinases as well, dysfunction of which would lead to longevity (*13-15*).

2. PAK1 versus TOR in our immune system and melanogenesis

Since then, a series of PAK1-blockers (as well as TOR-inhibitors) were identified or developed (*16-20*) for possible therapy of cancers (and all solid tumours) as well as many other PAK1/TOR-dependent diseases including hypertension (high blood pressure) and inflammation. However, there is a great problem (concern) around TOR-inhibitors, in particular rapamycin which was found to suppress/damage our "immune system" (for a review, *19*), while PAK1-blockers such as FK228 and curcumin activate our immune system (depending on both B- and T-cells) (*21,22*). Finally in 2017 we proved genetically that these B/T cell-dependent immune systems are normally suppressed by PAK1 (*23*, for a summary of PAK1 *vs*. TOR, see Figure 1). Naturally, since then, at least for cancer therapy, we focused our effort mainly on PAK1-blockers. However, screening PAK1-blockers by animal experiments would cost both money and time. Thus, during 2015-16, we finally

managed to device a "simple" cell culture system using melanoma cell line B16F10 in which the serum (PDGF)-dependent melanogenesis is inhibited by silencing PAK1 gene (*24,25*, for summary see Figure 1). Since this melanogenesis in cell culture is fortunately boosted by rapamycin or other TOR-inhibitors (*26,27*, for summary see Figure 1), we could easily exclude TOR-inhibitors, which suppress our immune system as well (see Figure 1).

Interestingly, however, regarding the so-called anti-cancer immune "check-point", rapamycin and PAK1-blockers share the "same" boat, namely both inhibiting PD-L1 expression in cancer cells (*28,29*), indicating that both TOR and PAK1 are essential for PD-L1 expression (*30,31*).

3. PAK1-blockers or TOR-inhibitors? That is the question

Rather ironically, however, we recently noticed that a few chemicals called "TOR-inhibitors" apparently inhibit melanogenesis as if they were PAK1-blockers (*32*, see Figure 1), while a new Gleevec derivative, called "nilotinib", which is supposed to block PAK1, promotes melanogenesis as if it were a TOR-inhibitor (*33,34*, see Figure 2).

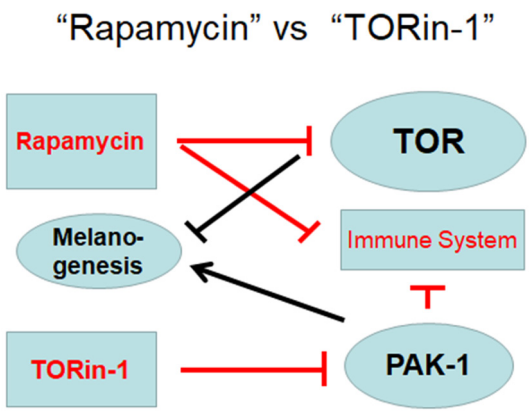

Figure 1. "TORin-1" blocks PAK1 instead of TOR in cells.

3.1. "TORin-1" developed as a TOR-inhibitor, blocks "PAK1"

As a so-called "side effect", rapamycin is known to inhibit the symptom of MS (multiple sclerosis), an auto-immune disease, as well as organ rejection, and therefore it has been marketed only as an "immune suppressor" since 1999. Although rapamycin is anti-oncogenic as well, it has never been recommended for the therapy of cancers or a rare benign tumour called TSC (tuberous sclerosis complex) which are mainly caused by abnormal activation of TOR.

Fortunately, a few other TOR-inhibitors have been developed in this century, which no longer cause this "side effect" of TOR, namely the immune-suppression. Among them is TORin-1 which was developed in 2010 by a group led by David Sabatini at Dana Farber Cancer Institute and MIT. This drug is an ATP-antagonist (*20*, see Figure 2), and directly inhibits the phosphorylation by both TORC1 and TORC2 with IC_{50} between 2 and 10 nM *in vitro*. In cell culture, however, its IC_{50} is around 250 nM, indicating that its "cell-permeability" might be rather poor (and clearly water-insoluble). Nevertheless, TORin-1 was effective at a dose of 20 mg/kg against the growth of a human U87MG (glioma) xenograft in mice. However, for its future clinical use, both cell-permeability and water-solubility of this chemical should be boosted somehow.

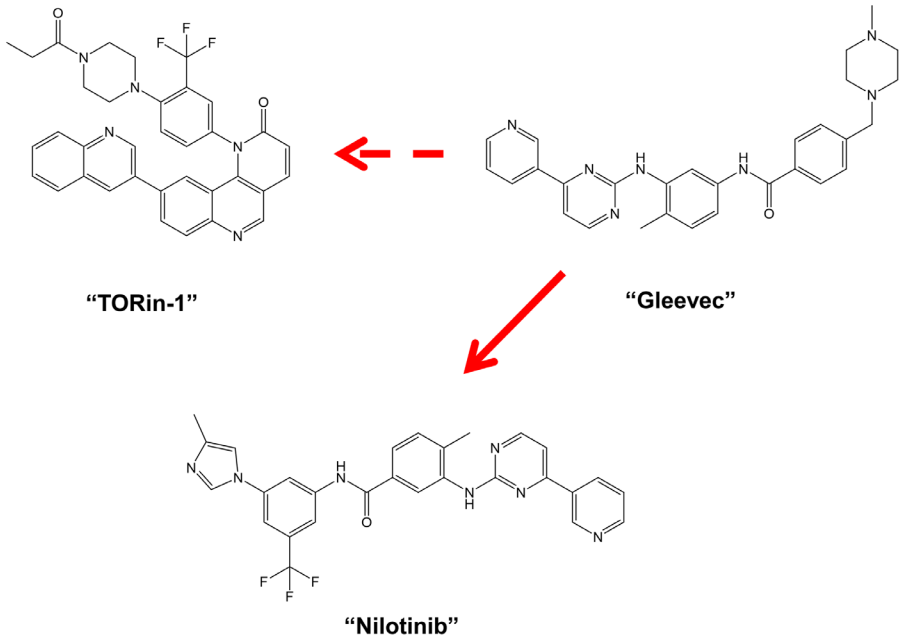

Figure 2. "TOR"-inhibitor or "PAK1"-blocker? That is the question.

The tumour-suppressor gene product called "Merlin" inhibits not only PAK1 directly (*35*), but also appears to inhibit TOR as well (directly or indirectly) (*36,37*), and the "loss-of-function" mutation of Merlin causes a rare benign tumour called NF2 (neurofibromatosis type 2), and shortens the lifespan of mice by 40% (from 2.5 years to 1.5 years). However, silencing PAK1 gene alone is sufficient to suppress the growth of NF2 tumours, and normalize the lifespan in mice (*15*).

Likewise, the tumour-suppressor complex of TSC1 and TSC2 appears to block not only TOR, but also PAK1 somehow (*38*), and the "loss-of-function" mutation of TSC1 or TSC2 causes a rare benign tumour called TSC. Rather surprisingly, the major target of TORin-1 in cells appears to be PAK1, instead of TORC1 or 2, mainly because it blocks "PAK1-dependent" melanogenesis (*32*), and shows no effect on immune system *in vivo*. Furthermore, according to 2014 article by US Team in Boston, prostaglandin synthesis by COX-2 whose gene expression depends on PAK1, was not affected by rapamycin, but significantly blocked by TORin-1, clearly indicating that (*i*) in addition to TOR, PAK1 is also abnormally activated somehow in TSC-deficient tumor cells, and (*ii*) TORin-1 blocks PAK1 at least in these TSC tumors (*38*). Interestingly, Gleevec and TORin-1 are chemically very similar (see Figure 2), but in terms of PAK1-blocking activity in cells, TORin-1 is still 40 times more potent than Gleevec in cells. Thus, TORin-1 could be potentially useful for all PAK1-dependent cancers or solid tumours including NF and TSC tumours for which no "effective" (FDA-approved) therapeutic has been available in the market so far.

3.2. "Nilotinib", developed as a "Gleevec" derivative, inhibits TOR *in vivo*

It also appears to be true in the "exactly" opposite direction. Gleevec was originally developed by Novartis team around mid-1990s mainly for the treatment of CML (Chronic Myeloid Leukemia), caused by abnormal activation of a Tyr-kinase called ABL. However, it was later found that Gleevec blocks PAK1 by inhibiting a few other Tyr-kinases such as PDGFR and c-KIT with IC_{50} around 10 μM in cells (for review, *39*, see Figure 2). Interestingly Gleevec treatment of melanocytes as well as CML patients causes a significant reduction of melanogenesis in cells or whitening of skins (*40*), clearly indicating the suppression of PAK1-dependent melanogenesis. Meanwhile, Gleevec treatment of CML patients also causes Gleevec resistance. Thus, around 2005, Novartis developed a new Gleevec derivative called "nilotinib" (see Figure 2), which works even on Gleevec-resistant CML. Nilotinib is a fluoride derivative of Gleevec (see Figure 2, *39*). However, just like TORin-1 which is also a fluoride compound, nilotinib appears to change its "major" target *in vivo*. Although nilotinib directly inhibits a series of Tyr-kinases including ABL and PDGFR *in vitro*, and eventually blocking PAK1 *in vivo*, nilotinib even at 5 μM appears to inhibit TOR as well *in vivo*, namely promoting melanogenesis in cell culture just like rapamycin (*33,34*). This "stunning" observation clearly indicates that nilotinib serves dominantly as a TOR-inhibitor in cells, instead of a PAK1-blocker. Thus, nilotinib could be potentially useful for both organ transplantation and MS therapy as well, just like rapamycin. Furthermore, the "main" reason why nilotinib is effective to "Gleevec-resistant" CML cells might not be due to its "*direct*" effect on a (Gleevec-resistant) ABL "mutant" in CML patients, but a "*side*" effect against TOR instead. In fact, according to the 2023 article (*https://www.mdpi.com/1422-0067/24/2/1234*) by an Italian team, TOR is abnormally activated in CML cells/*in vivo*, in particular under "hypoxia".

3.3. Pomalidomide, developed as a thalidomide analog

Thalidomide was best known as a "teratogenic" drug, which eventually caused the birth of so-called "thalidomide-children", after it is taken by pregnant women who suffer from "morning sickness".

Thereafter it was totally banned by FDA since the beginning of 1970s. However, ironically, it was found later (around 1990s) that if it is taken by "non-pregnant" people, it wouldn't cause any harmful "side effect". Instead, it was re-discovered as a "miracle" drug which cures a series of inflammatory or infectious diseases such as leprosy (*41*). However, thalidomide is known to suppress our immune system, just like rapamycin, strongly suggesting, if not proven as yet, that it inhibits TOR somehow (*42*). Yes, thalidomide stimulates "melanogenesis" (re-pigmentation) in hairs when old ladies (ca 75 years old) with "white" hairs are used for therapy of MM (Multiple Myeloma) or MS (Multiple Sclerosis) (*43*). Its rather complicated derivative called "lenalidomide" is also still melanogenic clinically (*44*, see Figure 3). Finally, a simple "amino" derivative of thalidomide called "pomalidomide" was developed (see Figure 3). So far pomalidomide showed neither melanogenic or immune-suppressive effect *in vivo*. Most

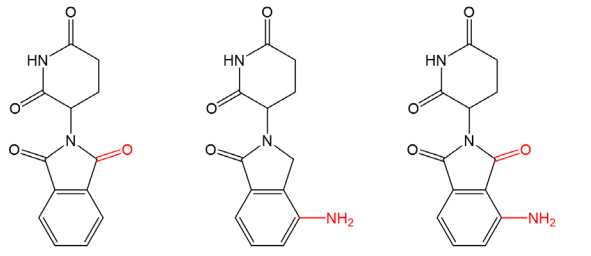

Figure 3. "Thalidomide" and its derivatives.

importantly, pomalidomide is no longer teratogenic even if it is taken by chick or fish embryos or pregnant persons (for a review, *45*).

According to the most recent reviews by experts on thalidomide and its derivatives, among their direct targets is a brain protein called "cereblon" (CRBN), and their binding to CRBN, a substrate recognition receptor for an E3 ubiquitin ligase (called "CRL4"), induces the recruitment of non-native substrates to CRL4CRBN and eventually their proteolytic degradation (*46*). In other words, thalidomide is among "PROTACs" which bind both E3 ubiquitin ligase(s) and their specific targets.

However, non-teratogenic pomalidomide still shows both "anti-angiogenic" and anti-cancer activities by ubiquitination-induced "proteolytic" degradation (so-called "PROTAC" action) of another target, a Tyr-kinase called "JAK", eventually blocking PAK1, just like thalidomide (*47*). Thus, it is now crystal-clear that the "teratogenesis" by thalidomide has nothing to do with its "anti-angiogenesis".

Finally we dug out a "possible" link of thalidomide to TOR (*48*): This drug activates somehow (but not through its direct action on CRBN) an anti-oncogenic kinase called "AMPK" (AMP-activated kinase), which activates the anti-oncogenic "TSC" complex, that eventually inhibits TOR.

In other words, pomalidomide is simply a PAK1-blocker, while thalidomide is a "CRBN/TOR" inhibitor as well as a PAK1-blocker. Very recently (2023), a Chinese group developed a potent TOR-specific PROTAC (protein-targeting chimera) by combining "POM" and a TOR-binding ligand called "MLN0128" (*49*), perhaps for their great fun, or at least proving that pomalidomide alone never inhibits (or binds) TOR. By the way, POM is 10 times more potent than thalidomide and lenalidomide for therapy of a hematological cancer called MM clinically, most likely because pomalidomide is no longer immune-suppressive (*50*).

4. Closing remarks

Although both PAK1 and TOR share the major roles in our life, namely "oncogenic" and "ageing", they play the "exactly opposite" roles in at least two other physiological functions, namely B/T-cell based immune system and PDGF-dependent melanogenesis. Thus, based on these differences, in particular their opposite roles in the melanogenesis of B16F10 melanoma cells, we are able to distinguish easily between PAK1-blockers and TOR-inhibitors. At least in three examples shown above (TORin-1, nilotinib, and pomalidomide), a "simple" modification of TOR-inhibitors (or PAK1-blockers) could result in their functional conversion to PAK1-blockers (or TOR-inhibitors) for so-called "chemical evolution", just like "penguins", which cannot fly in sky suddenly started swimming in sea with their "unique" feathers (called "flippers"), in order to reach their "new" home called "South Pole" (Antarctica) from their "old" homes (New Zealand or Australia) through the great ocean as soon as "vicious" cats were unfortunately introduced there (latter) by *Homo sapiens*.

Back in 2020, we briefly review three distinct PAK1-blockers whose anti-oncogenicity was potentiated 500-3,000 times for clinical application by a rather simple chemical modification (*51*). One of them is 1, 2, 3-triazolyl ester of ketorolac called "15K". This ester is more than 500 times "cell-permeable" than ketorolac which bears a COOH, and its anti-PAK1 activity also has been proven to be 500 times more potent than ketorolac in cell culture (*52*), and still remains to be "anti-melanogenic" (unpublished observation). However, although a CYP24-resistant derivative of vitamin D3 called "MART-10" was proven to be 1,000 times more potent than D3 as an anti-cancer agent in cell culture and *in vivo*, nobody knows whether MART-10 still remains as a PAK1-blocker (or TOR-inhibitor). Although D3 has been proven to block the RAC-PAK1 interaction (clearly being a PAK1-blocker) as is Ketorolac, to our surprise D3 has been recently reported to restore pigmentation in "vitiligo", and promote melanogenesis of B16F10 melanocytes, indicating the possibility that D3 might inhibit TOR as well (*53*). Thus, we are planning to test the effect of MART-10 on melano-genesis, determining if it blocks mainly PAK1 or TOR. By the way, according to 2018 clinical trial report from US and UK team (54), D3 (4,400 IU/d) stimulates both B-and T-cell based neonatal immunity of maternal supplementation, strongly suggesting that D3 is an immune promoter (mainly being a PAK1-blocker).

If we could give a "last" brief word (s) to readers, the whole "kinase" research world, hopefully for a further evolution, we would like to encourage them to do a cross-talk, or "horizontal"-thinking, instead of "vertical" thinking (as if they were mathematicians), breaking so-called "sectionalism". For an instance, it has been firmly established since 2010 that "AMPK-activators are PAK1-blockers", since the anti-oncogenic LKB1 was found to inactivate the oncogenic PAK1 by phosphorylating Thr at position 109, while it activates the anti-oncogenic AMPK by phosphorylating Thr at position 172 as well (*55*). Penguins learned how to swim by watching fishes, while flying fishes learned how to fly, by watching birds

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