Brief Report

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Several herbal compounds in Okinawa plants directly inhibit the oncogenic/aging kinase PAK1

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Summary **The p21-activated kinase 1 (PAK1) is emerging as a promising therapeutic target, and the search for blockers of this oncogenic/aging kinase would be potentially useful for the treatment of various diseases/disorders in the future. Here, we report for the first time the anti-PAK1 activity of compounds derived from three distinct Okinawa plants. 5,6-Dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK) from alpinia inhibited directly PAK1 more strongly than mimosine and mimosinol from leucaena. Cucurbitacin I isolated from bitter gourd/melon also exhibited a moderate anti-PAK1 activity. Hispidin, a metabolite of DK, strongly inhibited PAK1 with the IC_{50} = 5.7 μM. The IC_{50} of three hispidin derivatives (H1-3) for PAK1 inhibition ranges from 1.2 to 2.0 μM, while mimosine tetrapeptides [mimosine-Phe-Phe-Tyr (MFFY) and mimosine-Phe-Trp-Tyr (MFWY)] inhibit PAK1 at nanomolar level (IC_{50} of 0.13 and 0.60 μM, respectively). Thus, we hope these derivatives of hispidin and mimosine could be used as potential leading compounds for developing far more potent anti-PAK1 drugs which would be useful for clinical application in the future.**

Keywords: p21-activated kinase 1, 5,6-dehydrokawain, dihydro-5,6-dehydrokawain, mimosine, cucurbitacin I

1. Introduction

The family of p21-activated protein kinases (PAKs) belongs to Ras-related C3 botulinum toxin substrate/cell division control protein 42 (RAC/CDC42)-dependent serine/threonine kinases and in mammals consists of six distinct members (PAK1-6) (*1*). Among them, PAK2 and PAK4 are absolutely essential for the development of embryos (*1,2*). However, apparently PAK1 is not essential for embryogenesis, and PAK1-deficient mice look perfectly healthy, and are even resistant to inflammatory diseases, and PAK1-deficient mutant of *C. elegans* lives longer than the wild-type (*2*).

PAK1 is responsible not only for a variety of inflammatory diseases such as asthma and arthritis, but also for infection of HIV and influenza virus. In addition, PAK1 is essential for the growth of majority of solid tumors as well as their metastasis and angiogenesis (blood vessel formation required for the growth of solid tumors) (*1,2*). In other words, hyper-activation or over-expression of PAK1 would shorten the healthy lifespan, in part by causing cancers and a variety of other diseases/disorders such as diabetes (type 2), hyper-tension and Alzheimer's disease (*2*). Since PAK1 is not essential for the growth of normal cells (*2*), (unlike the conventional anti-cancer drugs) blocking PAK1 *per se* does not cause any side effect. Thus, selective small molecule PAK1-blockers (natural or synthetic) would have a potentially huge market value for the treatment of a variety of PAK1-dependent diseases/disorders and the longevity as well in the future. Furthermore, considering the well-known fact that people in Okinawa have enjoyed the longest healthy lifespan among Asian population, we recently got interested in testing the possibility that foods or

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herbal products derived from some of plants uniquely grown in Okinawa might contribute to the longevity among Okinawa people by blocking PAK1.

Leucaena leucocephala and *Alpinia zerumbet*, distributed widely in Okinawa in particular and in subtropical and tropical zones in general, have lent them as multi-purpose plants such as the development of novel cosmetics and medicines (*3*). Alpinia is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties (*4*). The essential oil from its leaves possesses relaxant and anti-spasmodic actions in rat ileum (**5**). Early data have indicated that mimosine, 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK), which are major ingredients in leucaena and alpinia, are shown to have various biological activities including anti-fungal, anti-inflammatory, anti-tumor and anti-viral (*6,7*). In our laboratory, we have prepared several compounds from leucaena and alpinia against HIV-1 integrase and neuraminidase enzymes (*7*), for inhibition of advanced glycation end products and for prevention of the skin diseases (*8,9*). Bitter gourd (*Momordica charantia*), known as "goya" in Japan, has been implicated in different pharmacological activities such as anti-diabetic, anti-bacterial, anti-viral, anti-cancer, and anti-obesity (*10*). In spite of given diverse biological activities, their anti-PAK1 activity still remained unknown. In this study, we first focused primarily on the potential anti-PAK1 activity of compounds isolated from alpinia, leucaena, and bitter gourd. We then prepared several derivatives from these natural compounds for further potentiation of their anti-PAK1 activity (Figure 1). To the best of our knowledge, this is the very first report showing the direct inhibition of PAK1 by compounds derived from these three Okinawa plants.

2. Materials and Methods

2.1. *Chemicals and reagents*

Tris(triethylsilyl)silane, resveratrol were obtained from Sigma-Aldrich (Shinagawa-ku, Tokyo, Japan) whereas trifluoromethanesulfonic acid was obtained from Nacalai Tesque (Nakagyo-ku, Kyoto, Japan). Curcumin was purchased from Kanto Chemical Co. (Chuo-ku, Tokyo, Japan). Fmoc-L-amino acids were purchased from Hipep Laboratories (Kamigyo-ku, Kyoto, Japan).

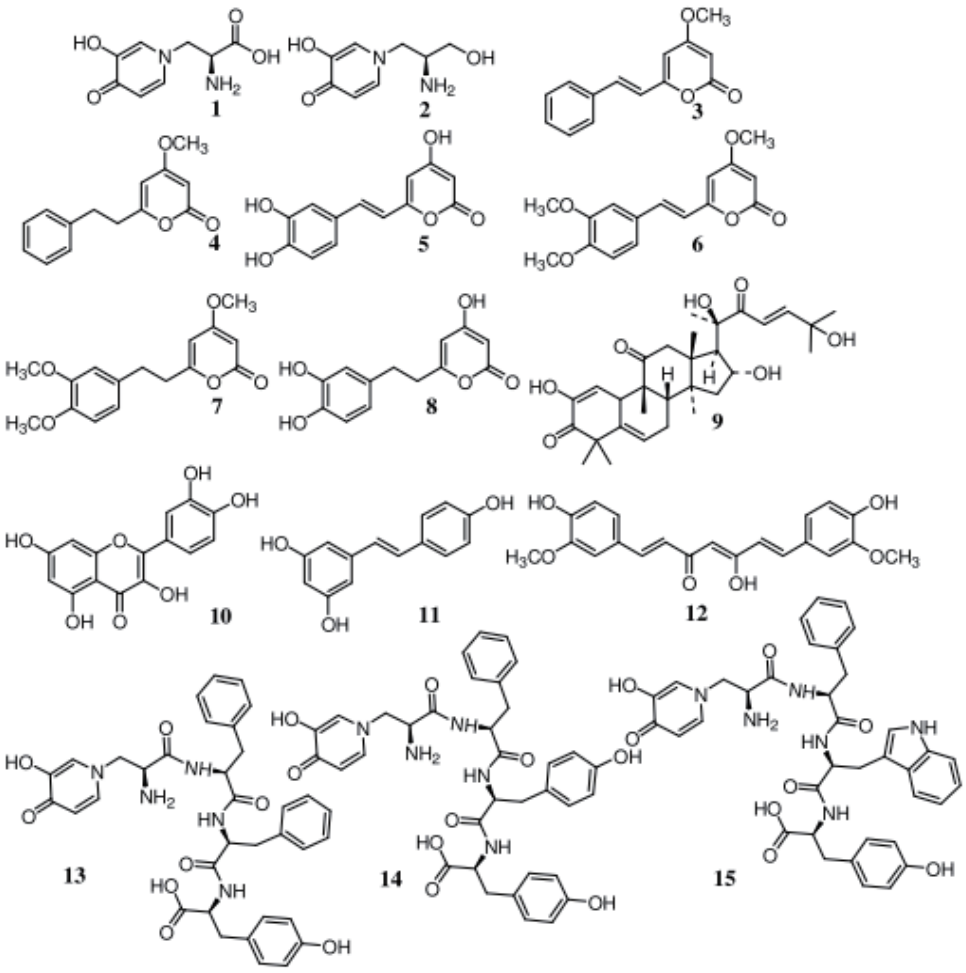

Figure 1. Chemical structures of compounds. Mimosine (**1**), mimosinol (**2**), DK (**3**), DDK (**4**), hispidin (**5**), H1 (**6**), H2 (**7**), H3 (**8**), cucurbitacin I (**9**), quercetin (**10**), resveratrol (**11**), curcumin (**12**), MFFY (**13**), MFYY (**14**), MFWY (**15**).

2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Novabiochem (Schuchardt, Hohenbrunn, Germany). Quercetin, wang resin (1% DVB), N,N'-diisopropylcarbodiimide (DIC), N,N'-diisopropylethylamine (DIEA), and 1-hydroxy-1*H*-benzotriazole (HOBt) were purchased from Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan). Unless otherwise mentioned, all reagents were of analytical grade and were obtained from Wako Pure Chemical Industries and Kanto Chemical Co. The ^{1}H spectra were recorded on a JEOL JNM-ECA400 (Tokyo, Japan). Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane (TMS).

2.2. *Mimosine isolation from Leucaena leucocephala leaves*

Samples of *Leucaena leucocephala* leaves were collected at the Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan (lat 26ºN, long 127ºE). Fresh leaves (1.5 kg) were boiled in 5 L water for 10 min. The cooled liquid extract was sieved by suction filtration in a shaking bath (As One, Nishi-ku, Osaka, Japan) and the filtrate was mixed with ion-exchange resin (2 kg), stirred for 30 min, and left overnight. The resin was rinsed with distilled water 5-6 times and 5 L 80% ethanol was added dropwise to remove chlorophyll. Mimosine was dissolved from the resin with dropwise addition of 6 L of 2 N NH_4OH. The liquid extract was concentrated to a final volume of 300 mL at 40ºC under reduced pressure. The solution was adjusted to pH 4.5-5.0 with 6 N HCl and mimosine was precipitated at 4ºC overnight. The precipitate was recrystallized from 5 N NaOH (pH 9.0) and 6 N HCl (pH 4.5-5.0) and then allowed to stand at 4ºC to give pure mimosine. Mimosine was stored at –20ºC until further use (*11*).

2.3. *Preparation of mimosinol from mimosine*

Trifluoromethanesulfonic acid (187 µL, 2 mmol) was added to a 25-mL round-bottom flask containing 3.4 mL dichloromethane (CH_2Cl_2). After stirring at room temperature, tris(triethylsilyl)silane solution (618 µL, 2 mmol) was added dropwise and the mixture was stirred at room temperature for 3 h until the solution become clear. Mimosine (0.4 g, 2 mmol) was placed in a round-bottom flask, to which imidazole (0.15 g, 2.2 mmol) and dimethylformamide (DMF):CH_2Cl_2 (3.4 mL, 1:1) were then added. The reaction flask was cooled to 0ºC and tris(triethylsilyl)silyl triflate was added dropwise. After the addition was completed, the reaction was stirred at room temperature for 2 h. Mimosine ester was obtained from the filtrate by evaporation. A solution of sodium borohydride (0.28 g, 7.2 mmol) in 3 mL 50% ethanol was added to solution of mimosine ester in 3 mL of 50% ethanol. The resulting mixture was refluxed at room temperature for 5.5 h and the solvent ethanol was evaporated in vacuo. The aqueous solution thus obtained was extracted with ethyl acetate (3 × 20 mL); the combined extracts were washed with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated to give mimosinol as a colorless crystal (352 mg, 95% yield) (Figure 2). ^{1}H NMR (D_2O, 400 MHz) δ 7.93 (s, 1H, CH), 7.28 (s, 1H, CH), 3.02-2.86 (d, 2H, CH), 2.08-1.91 (s, 2H, CH_2), 1.58-1.54 (m, 2H, CH_2), 1.22-1.11 (m, 1H, CH).

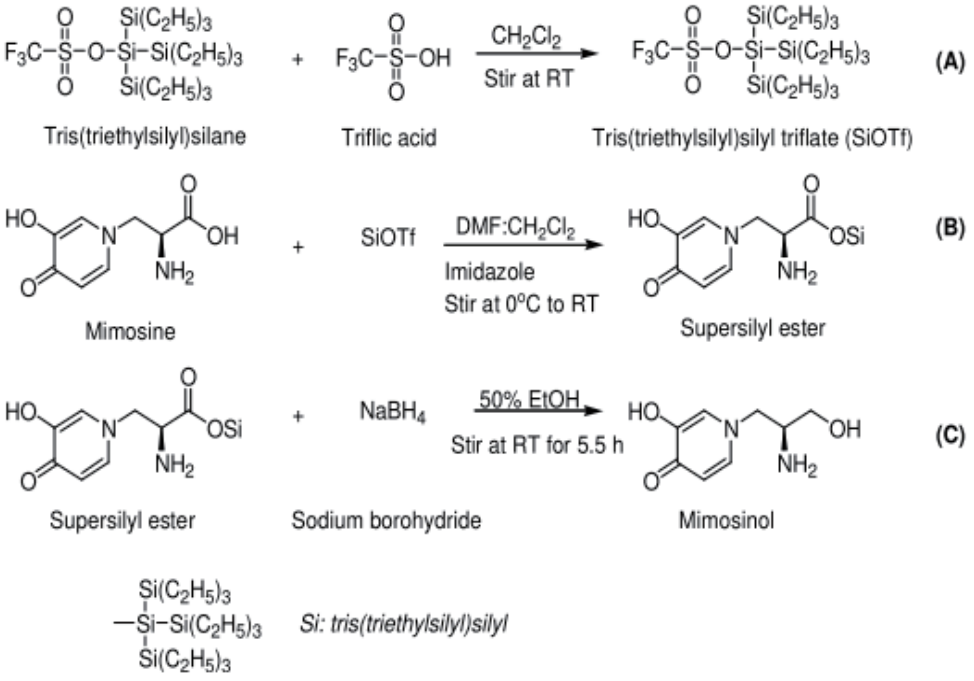

Figure 2. Route for reduction of mimosine to mimosinol. (A) *In situ* generation of tris(triethylsilyl)silyl triflate, **(B)** Installation of tris(triethylsilyl)silyl group into mimosine, **(C)** Reduction of mimosine ester to mimosinol using sodium borohydride.

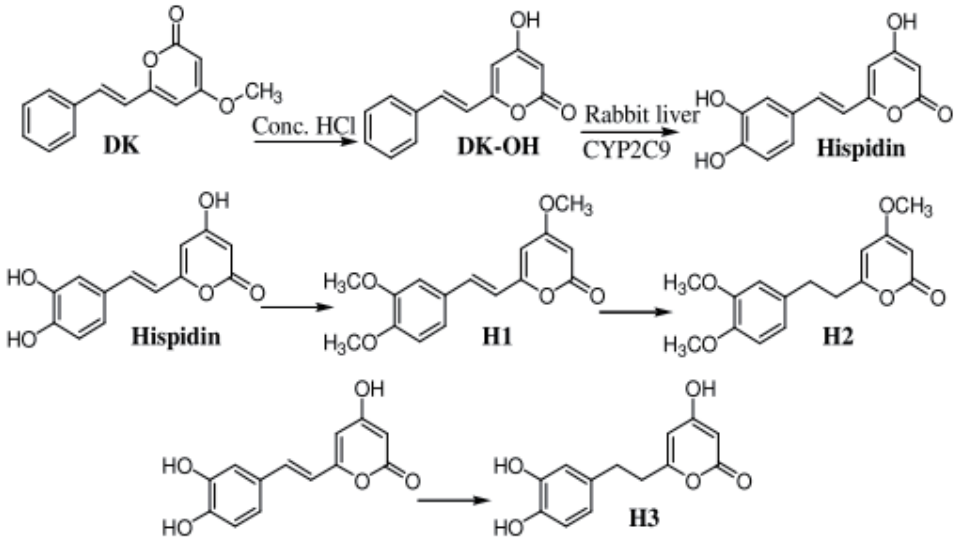

Figure 3. Preparation of hispidin and their derivatives. Bioconversion of 5,6-dehydrokawain (DK) to hispidin by CYP2C9 in the microsomes of rabbit liver. Hispidin derivatives (H1-3) were prepared from hispidin.

2.4. *Synthesis of mimosine tetrapeptides*

Compounds were prepared according to procedure described previously (*12*). In brief, Fmoc-L-amino acid (1.6 mmol) was dissolved in 5 mL of dimethylacetoamide, followed by adding HOBt (1.6 mmol) and DIC (1.6 mmol). The mixture was added to swollen Wang resin (1 g) in DMF and stirred for 17 h. After deprotection of 9-fluorenylmethoxycarbonyl (Fmoc) with 25% piperidine, the next amino acids were coupled to the resin mixture solution (Fmoc-amino acid/HOBt/HBTU/DIEA = 4:3:3.6:8) and stirred for 1 h to form tripeptides. The Kaiser's test was used to assess the completeness of the coupling reaction. After final coupling with mimosine, the final cleavage was performed by shaking the resin vigorously in 95% trifluoroacetic acid (TFA) for 1 h. The resin was filtered and washed with TFA. The obtained filtrate was precipitated with ice-cold diethyl ether. The resulting precipitate was filtered, washed with diethyl ether, and dried under a vacuum to obtain the desired mimosine tetrapeptides. The purified compounds were evaluated using LC-MS (ESI): *m/z* $[M-H]^+$ 693.2, 670.1, 654.2 for MFWY, MFYY, and MFFY, respectively.

2.5. *Isolation of DDK and DK compounds from Alpinia zerumbet*

The rhizomes of alpinia were collected from the University of the Ryukyus campus, Okinawa, Japan. The rhizomes (2 kg) were boiled in 10 L water for 20 min. The solution was cooled at room temperature and sieved by suction filtration (As One, Nishi-ku, Osaka, Japan). The filtrate was reduced to 1 L under vacuum at 40°C, and extracted with hexane (3 × 500 mL). The organic layer was evaporated to complete dryness under vacuum. The dried crude extract was boiled in water and filtered hot. The residue obtained was purified by high-performance liquid chromatography (HPLC) (Shimadzu, Nakagyo-ku, Kyoto, Japan) to give DK. The filtrate was crystallized at 4°C, and the crystals were purified further using HPLC to obtain DDK. For purification of DDK and DK, the major peaks were collected using mobile phase including solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol). The gradient elution was performed as follows: 1-10 min, isocratic 50% B; 10-20 min, linear gradient 50-100% B; 20-30 min, isocratic 100% B; 30-35 min, linear gradient 100-50% B. The flow rate and absorbance wavelength were set at 0.8 mL/min and 280 nm, respectively (*7*).

2.6. *Preparation of hispidin and cucurbitacin I compounds*

These two compounds were prepared by other group in our laboratory. DK was converted to hispidin by cytochrome P450 2C9 enzyme (CYP2C9) in the microsomes of rabbit liver (Figure 3) (*13*). Cucurbitacin I was isolated from Okinawa bitter gourd (*Momordica charantia*) fruits.

2.7. *Preparation of hispidin derivatives (H1-3)*

Hispidin (3 mg) was dissolved in 0.6 mL methanol:CH_2Cl_2 (1:5). The solution was cooled to 0°C, and 0.5 mL of diazomethane in CH_2Cl_2 was added. The mixture was stored overnight at 4°C. Solvents were evaporated, and the residue was purified by silica gel preparative thin layer chromatography (PTLC) to obtain H1 as pale yellow powder (2 mg, 67% yield) (*14*). Compound H1 (3.5 mg) dissolved in 0.82 mL of MeOH:$CHCl_3$ (1:1) was stirred for 2 h in the presence

of 10% of Pd/C (0.65 mg). The mixture was filtered and solvent was evaporated in vacuo. Purification was achieved by column chromatography to afford compound H2 as a white solid (3 mg, 85%) (*15*). The similar procedure was used to prepare H3 from hispidin.

Data for 6-(3,4-dimethoxystyryl)-4-methoxy-2H-pyran-2-one (H1). ^{1}H NMR (CDCl3, 400 MHz) δ 7.43 (d, 1H, CH), 7.07 (dd, 1H, CH), 7.00 (d, 1H, CH), 6.85 (d, 1H, CH), 6.43 (d, 1H, CH), 5.89 (d, 1H, CH), 5.46 (d, 1H, CH), 3.91 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3).

Data for 6-(3,4-dimethoxyphenethyl)-4-methoxy-2H-pyran-2-one (H2). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.77 (d, 1H, CH), 6.69 (dd, 1H, CH), 6.66 (d, 1H, CH), 5.69 (d, 1H, CH), 5.40 (d, 1H, CH), 3.84 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 2.91 (m, 2H, CH_2), 2.71 (m, 2H, CH_2).

Data for 6-(3,4-dihydroxyphenethyl)-4-hydroxy-2H-pyran-2-one (H3). ^{1}H NMR (DMSO, 400 MHz) δ 7.29 (d, 1H, CH), 7.20 (dd, 1H, CH), 6.76 (d, 1H, CH), 6.11 (d, 1H, CH), 5.26 (d, 1H, CH), 3.34 (m, 2H, CH_2), 2.99 (m, 2H, CH_2).

2.8. *In vitro assay for the kinase PAK1*

Its kinase activity was measured by ADP-Glo™ kinase assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions. Human PAK1 (10 µL) at 25 ng/reaction concentration was incubated with 5 µL of test compounds at various concentrations for 10 min. The kinase reaction was started by the addition of 2.5 X adenosine triphosphate (ATP)/substrate mix (10 µL) which was incubated for 40 min. The reaction was terminated by 25 µL ADP-Glo™ reagent, followed by 40 min incubation. To this reaction mixture was added 50 µL of the kinase detection reagent which converts adenosine diphosphate (ADP) to ATP that eventually generates a luciferin/luciferase-based fluorescence. After 30 min incubation, luminescence was recorded by MTP-880Lab microplate reader (Corona, Hitachinaka-ku, Ibaraki Japan) with an integration time of 0.5 s per well. Blank wells lacked the test compounds and PAK1 but did include all remaining components. All procedure steps were conducted at room temperature. The percentage inhibition was calculated relative to the control kinase activity without any inhibitor.

3. Results and Discussion

In previous study, we discovered that DK and DDK from alpinia rhizomes as well as mimosine from leucaena leaves are strong inhibitors of HIV-1 integrase and neuraminidase activity (*7,12*). Interestingly, PAK1 is essential for the replication of several viruses including HIV and influenza virus (*2*). In this study we found that DK, DDK and mimosine compounds inhibited directly the kinase activity of PAK1. As summarized in Table 1, the anti-PAK1 activity of DK and DDK is significantly better than mimosine and mimosinol. The IC_{50} values of DK and DDK are 17 and 10 µM, respectively, while mimosine and mimosinol had the IC_{50} values of 37 and 30 µM, respectively. Furthermore, a metabolite of DK called hispidin had a strong anti-PAK1 activity (IC_{50} = 5.7 µM), almost equivalent to curcumin (IC_{50} = 7.0 µM), but clearly stronger than resveratrol (IC_{50} = 15 µM). Considering that one of the benzene rings in resveratrol is simply replaced by an α-pyrone ring in hispidin, it is likely that α-pyrone significantly contributes to an increase in the anti-PAK1 activity. However, Upadhyay *et al.* (7) suggested that the methoxy group at C-5 of DDK and DK could be the major contributor to their anti-HIV activity. Thus, perhaps this methoxy group of DK and DDK could also be attributed to their anti-PAK1 activity, in addition to the α-pyrone ring. In comparison of the anti-PAK1 activity between DK and hispidin, *in vivo* DK could act as a PAK1 inhibitor in two ways, first as DK itself and second after it is converted to hispidin by the enzyme CYP2C9. The anti-PAK1 activity of DK is significantly weaker than its metabolite "hispidin". Thus, it is most likely that the attachment of two OH groups to the benzene ring of DK or DDK contributes to an increase in the anti-PAK1 activity. We further prepared a few hispidin derivatives in an attempt to potentiate their anti-PAK1 activity. As shown in Table 1, the two methoxy derivatives (H1-2) inhibited PAK1 more strongly than hispidin, with the IC_{50} values ranging from 1.2 to 1.6 µM.

Table 1. Anti-PAK1 activity of herbal compounds and their derivatives

compound	IC_{50} (µM) for PAK1 inhibition
mimosine	37
mimosinol	30
MFFY	0.13
MFYY	2.3
MFWY	0.60
DK	17.1
DDK	10.3
hispidin	5.7
H1	1.6
H2	1.2
H3	2.0
cucurbitacin I	19
quercetin	340
resveratrol	15
curcumin	7.0

The IC_{50} values were determined graphically as the concentration of each compound that showed 50% inhibitory activity. M: mimosine, F: phenylalanine, W: tryptophan, Y: tyrosine.

The anti-PAK1 activity of a few mimosine tetrapeptides was also evaluated. Interestingly, at least two of these peptides inhibited PAK1 at nanomolar level. MFFY and MFWY (IC_{50} = 0.13 and 0.60 µM, respectively) were more potent than MFYY (IC_{50} = 2.3 µM).

Cucurbitacins, a family of tetracyclic triterpenes, are used in folk and traditional medicine which have selective biological properties against carcinogenesis. Recently, it has been suggested as anti-inflammatory and anti-cancer agents (*16*). Cucurbitacin I is a triterpenoid compound bearing diverse physiological actions such as inducing apoptosis, blocking cell cycle, inhibiting tyrosine kinase JAK2 (*17*). Since JAK2 is responsible for the activation of PAK1, this triterpenoid could block PAK1 at least indirectly in cells (*18*). By isolating cucurbitacin I from bitter gourd, we showed for the first time that in fact this compound directly inhibited PAK1 with the IC_{50} of 19 μM.

By the simple method used in our laboratory, these pyrones (DK, DDK) as well as mimosine and mimosinol can be prepared easily from alpinia and leucaena. Moreover, the synthesis of their derivatives is not costly; thus, in theory, their further chemical modification for creating more potent PAK1-inhibitors could be economically feasible.

In conclusion, we showed for the first time that compounds prepared from alpinia, leucaena and bitter gourd directly inhibit PAK1. Although these herbal compounds *per se* are not potent PAK1 inhibitors in comparison with curcumin, we managed to potentiate significantly their anti-PAK1 activity by specific chemical modification. For an instance, the mimosine tetrapeptide MFFY inhibits PAK1 with the IC_{50} around 100 nM. So far the most potent PAK1-specific inhibitors are FRAX486 and FRAX597 with the IC_{50} around 10 nM, although both their cell-permeability and water-solubility/bioavailability are rather poor (*19*). Thus, we hope that the "MFFY" in particular could serve as the first leading compounds for the development of far more potent and water-soluble anti-PAK1 derivatives which would be useful for clinical application in the future. Furthermore, we are currently measuring their anti-protein kinase B (AKT) activity, to make it sure that they would not cause any side effect *in vivo*, as unlike PAK1, the oncogenic kinase AKT is known to be required for the growth of normal cells, in particular heart development as well. AKT-deficient mice are embryonically lethal, mainly due to the heart failure (*2*).

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