Brief Report

DOI: 10.5582/ddt.2017.01011

Frondoside A from sea cucumber and nymphaeols from Okinawa propolis: Natural anti-cancer agents that selectively inhibit PAK1 *in vitro*

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Summary **A sulfated saponin called "Frondoside A" (FRA) from sea cucumber and ingredients from Okinawa propolis (OP) have been previously shown to suppress the PAK1-dependent growth of A549 lung cancer as well as pancreatic cancer cells. However, the precise molecular mechanism underlying their anti-cancer action still remains to be clarified. In this study, for the first time, we found that both FRA and OP directly inhibit PAK1 *in vitro* in a selective manner (far more effectively than two other oncogenic kinases, LIMK and AKT). Furthermore, at least two major anti-cancer ingredients of OP, nymphaeols A and C, also directly inhibit PAK1 *in vitro* in a selective manner. To the best of our knowledge, FRA is the first marine compound that selectively inhibits PAK1. Likewise, these nymphaeols are the first propolis ingredients that selectively inhibit PAK1.**

Keywords: Propolis, sea cucumber, frondoside A, nymphaeols, PAK1, cancers

1. Introduction

Since conventional chemotherapeutics such as DNA/microtubule poisons cause serious side effects such as hair-loss and suppression of immune response, recently cancer patients, in particular those who suffer from formidable pancreatic or lung cancers, started seeking an alternative approach for cancer therapy by using natural remedies that do not cause any serious side effect. A bee product called "propolis" has been used as one of these herbal cancer therapeutics for last three decades. Two major propolis products available in the market are ARC (artepillin C)-based Brazilian green propolis (GP) and CAPE (caffeic acid phenethyl ester)-based propolis called Bio 30 or Bio 100 from New Zealand (*1-3*). However, recently, propolis from subtropical regions such as Okinawa, Taiwan, and Hawaii has been studied extensively, mainly because of its unique ingredients such as geranylated flavonoids (nymphaeols) (*4-6*). Very recently, we found that Okinawa propolis (OP) is highly anti-angiogenic *in ovo* (fertilized eggs), clearly several times more potent than GP as a herbal anti-cancer remedy, and blocks the oncogenic/ageing kinase PAK1 at least in cell culture (*4,7*). Furthermore, we confirmed that OP is a potent elixir extending the healthy lifespan of C. elegans (*7*).

In addition to these three distinct propolis products, the potent anti-cancer activity of a sulfated saponin called "frondoside A" (FRA) from an edible sea cucumber (*Cucumaria frondosa*) has recently drawn much attention of pancreatic and lung cancer patients. According to previous studies by Thomas Adrian and others, FRA inhibits the growth of A549 lung cancer and pancreatic cancer cells with IC_{50} ranging 1-3 μM in cell culture, and up-regulates the tumor-suppressor p21, an inhibitor of CDKs (cyclin-dependent kinases) (*8,9*). *In vivo* (xenograft in nude mice) FRA (1 mg/kg/day, *i.p.*) significantly suppresses the growth of human pancreatic cancer (9). We have shown previously that the expression of p21 gene is suppressed by PAK1 (*10*). Furthermore,

Released online in J-STAGE as advance publication April 24, 2017.

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since both A549 lung cancer and pancreatic cancer cells carry the oncogenic Ki-RAS mutant, and their growth depends on PAK1, it would not be unreasonable for us to suspect that FRA might block the oncogenic/ageing kinase PAK1 somehow. Here, we have confirmed that both OP and FRA as well as nymphaeols directly inhibit PAK1 *in vitro* in a selective manner.

2. Materials and Methods

2.1. *Chemicals and reagents*

Human lung cancer cell line A549 was purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Alcohol extract of Okinawa propolis (OP) was prepared as previously described (*7*). Nymphaeols were isolated from OP through HPLC as previously described (*4*). Frondoside A (FRA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both recombinant PAK1 and LIMK were obtained from SignalChem Pharmaceuticals Inc. (Richmond, British Columbia, Canada).

2.2. *Assay for anti-cancer activity in cell culture*

The number of viable cells after treatment with either frondoside A or nymphaeols (see Figure 1 for their chemical structures) was measured by Trypan blue assay in a hemocytometer as described previously (*11*). Briefly, A549 lung cancer cells (2×10^5 cells/well) were seeded for 24 h, and then treated with various test compounds at the indicated concentrations for 72 h, and the number of viable (unstained) cells were counted after Trypan blue staining.

2.3. *In vitro anti-kinase (PAK1/LIMK/AKT) assay*

The kinase activity of PAK1, LIMK, and AKT was measured *in vitro* by ADP-Glo kinase assay kit (Promega, Madison, WI, USA), according to the manufacturer's instruction, as previously described (*12*). Briefly, the recombinant human PAK1 (10 ng) or LIMK (10 ng) per reaction was treated with either OP, FRA or nymphaeols at the indicated concentrations in the presence of ATP, with either myelin basic protein (MBP) for PAK1 assay or cofilin for LIMK assay as their protein substrates, during the 40 min *in vitro* kinase reaction. Then the reaction was terminated with the ADP¬-Glo reagent. In the case of AKT assay, instead of using the recombinant AKT, A549 lung cancer cells were cultured for 48 h, and cell lysates were immuno-precipitated (IP) with anti-AKT IgG in the presence of protein A-agarose beads (*11*), and the resultant AKT in IP was treated with various test compounds at the indicated concentrations in the presence of ATP and MBP during 40 min *in vitro* kinase assay, which was then terminated with the ADP-

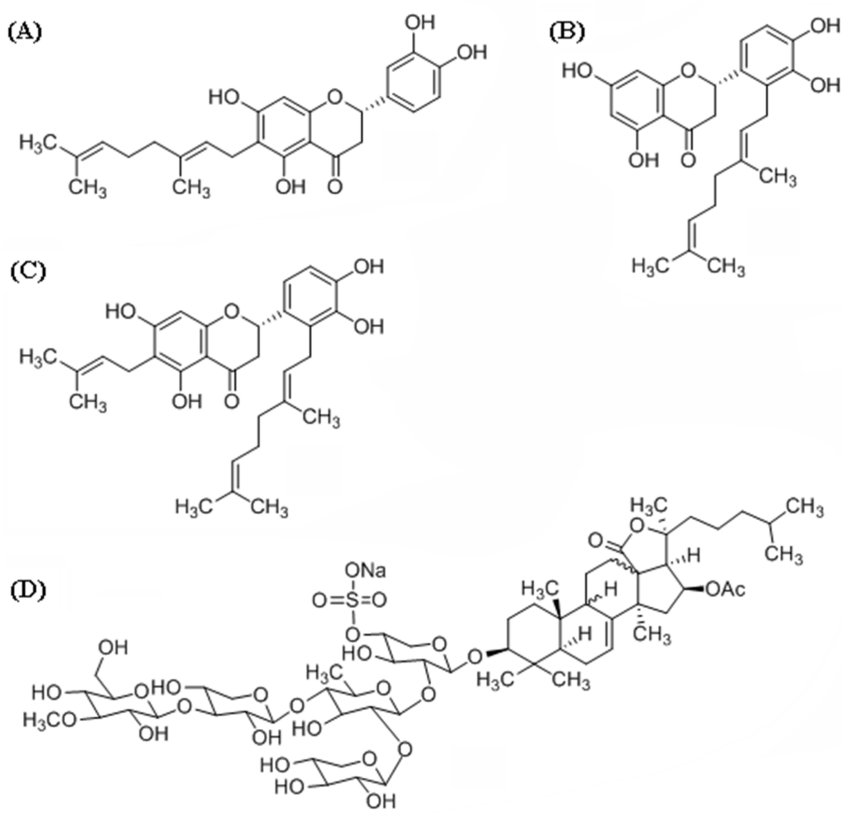

Figure 1. Chemical structures of nymphaeols A-C and frondoside A. (A) nymphaeol A; **(B)** nymphaeol B; **(C)** nymphaeol C; **(D)** frondoside A.

Glo reagent. To these reaction mixtures was added the kinase detection reagent that converts ADP to ATP which eventually generates a luciferin-luciferase based fluorescence.

2.4. *Statistical 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. *Okinawa propolis (OP) and its major ingredients directly inhibit PAK1 in vitro*

3.1.1. *Okinawa propolis (OP) directly inhibits PAK1 in vitro*

We have previously found that Okinawa propolis (OP) inhibits the PAK1-dependent growth of A549 lung cancer cells with IC_{50} around 12 µg/mL, while it blocks PAK1 in the same cell culture with the apparent IC_{50} around 6 µg/mL as judged by "Macaroni-Western" ATP-Glo kinase assay (*7*). Generally speaking, when a compound blocks PAK1 by inhibiting an upstream activator of PAK1 such as RAC, instead of directly inhibiting PAK1, the apparent anti-PAK IC_{50} value is usually 3-4 times higher than the anti-cancer IC_{50} value (*11*). Since the outcome with OP is clearly opposite, the possibility rose that OP might directly inhibit PAK1. Here we have confirmed this notion. As shown in Figure 2, OP directly inhibited the recombinant PAK1 *in vitro* with IC_{50} around 10 µg/mL.

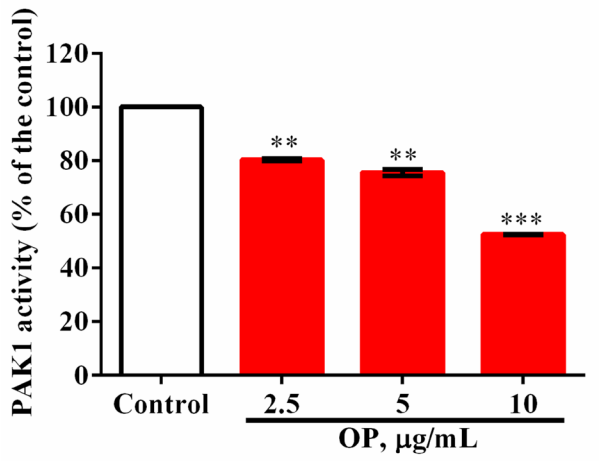

Figure 2. Okinawa propolis (OP) directly inhibits PAK1 *in vitro*. Recombinant PAK1 from SignalChem was treated with OP at the indicated concentrations *in vitro*. The experiments are conducted with twice, and the results are mean ± SE. IC_{50} of OP against PAK1 is around 10 µg/mL. Asterisks on each bar indicate significant differences between treatment and control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.1.2. *Anti-cancer activity of nymphaeols A and C from OP*

The major anti-cancer ingredients in OP are geranylated flavonoids called nymphaeols A-C (*5*), and at least nymphaeol A has been shown to inhibit the PAK1-dependent angiogenesis *in ovo* (fertilized eggs) (*4*), suggesting that it could block PAK1 somehow. Here, we have tested the anti-cancer activity of nymphaeols A and C. As shown in Figure 3, nymphaeols A and C inhibit the growth of A549 cancer cells with the $IC_{50} = 4$ µM and 7 µM, respectively.

3.1.3. *Anti-PAK1 activity of nymphaeols A and C in vitro*

The next, we have tested the anti-PAK1 activity *in vitro*. As shown in Figure 4, like OP, both nymphaeols A and C directly inhibited PAK1 with IC_{50} around 10 µM.

3.1.4. Kinase specificity of nymphaeols

In order to determine how selective the direct action of

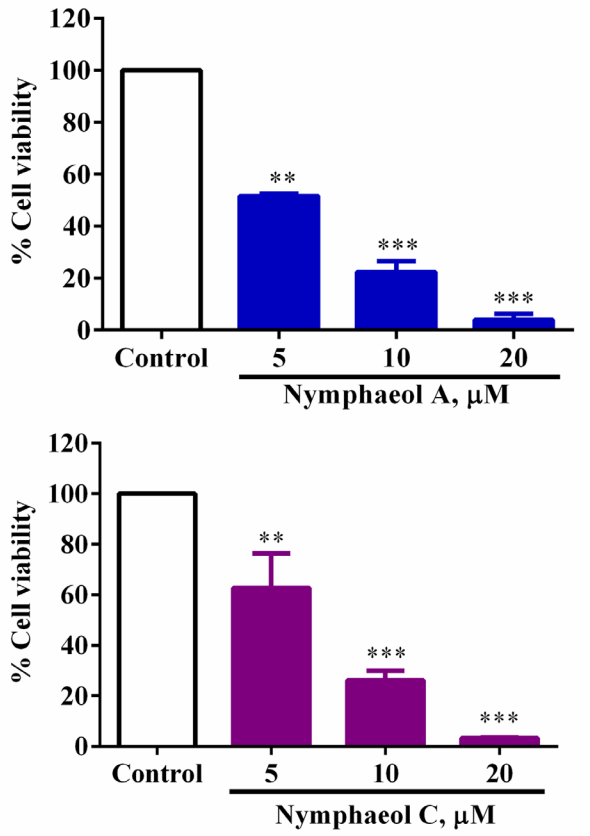

Figure 3. Anti-cancer activity of nymphaeols against the growth of A549 lung cancer cells. A549 cells were treated with either nymphaeols A (left) or C (right) at the indicated concentrations for 72 h, and the number of the viable cells was counted by Trypan blue staining. The results are mean ± SE of two independent experiments. IC_{50} of nymphaeols A and C are around 4 and 7 µM, respectively. Asterisks on each bar indicate significant differences between treatment and control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

nymphaeols towards PAK1 is, we have tested their anti-LIM kinase (LIMK) and anti-AKT activity *in vitro* as well. As summarized in Table 1, both nymphaeols A and C inhibited LIMK and AKT, but with the far higher IC_{50} (160 µM and 170 µM against LIMK, and 42 µM and 74 µM against AKT, respectively), confirming their specificity towards PAK1.

3.2. *Anti-PAK1 activity of frondoside A (FRA)*

Extracts of several distinct sea cucumbers have been shown to suppress the growth of cancer cells including A549 lung cancer and pancreatic cancer cell lines which carry the oncogenic Ki-RAS mutant (*8,9*). In most cases, anti-cancer ingredients derived from sea cucumbers belong to sulfated saponins. Among these saponins, frondoside A (FRA) from *Cucumaria frondosa* is the most potent so far, inhibiting the PAK1-dependent growth of A549 cancer cells with IC_{50} around 2.5 µM for 24 h (*9*), but under our own culture conditions (72 h), the IC_{50} against A549 is around 0.6 µM (see Table 1). However, the precise molecular mechanism underlying its anti-cancer action still remains to be clarified.

A few previous observations raised the possibility that FRA might inhibit PAK1 directly (or indirectly): (*i*) FRA inhibits the PAK1-dependent growth of A549 cancer cells, (*ii*) FRA up-regulates p21 (CDK inhibitor) whose expression is suppressed by PAK1 (*8-10*), and (*iii*) nymphaeols from OP directly inhibits PAK1 in a selective manner (see Figure 4 and Table 1). Thus, we were prompted to test *in vitro* if FRA could inhibit PAK1 and a few other kinases as well. As shown in Figure 5, FRA directly inhibits PAK1 *in vitro* with IC_{50} around 1 µM, but both LIMK and AKT with IC_{50}

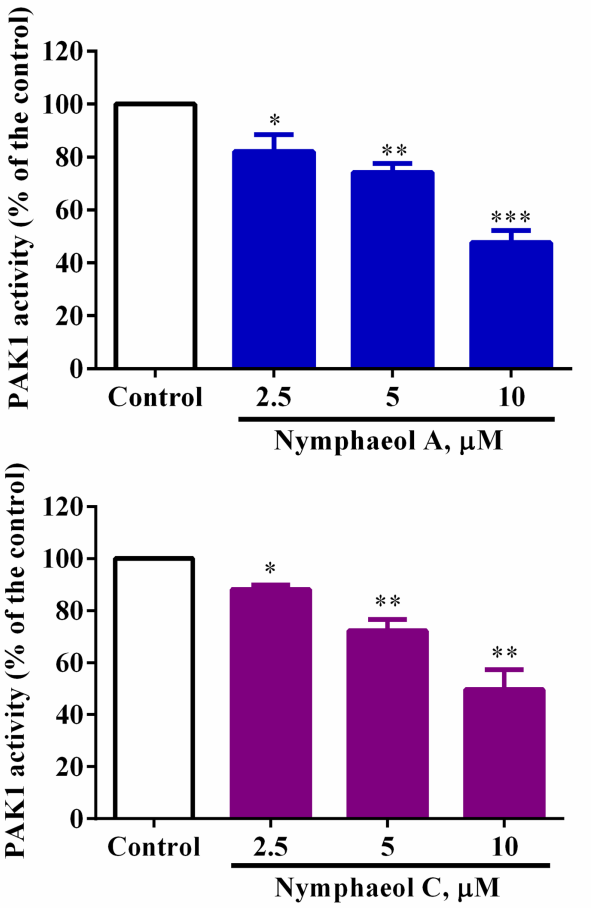

Figure 4. Nymphaeols directly inhibit PAK1 *in vitro*. PAK1 was treated *in vitro* with either nymphaeol A (left) or C (right) at the indicated concentrations. IC_{50} of both nymphaeols A and C is around 10 µM. Asterisks on each bar indicate significant differences between treatment and control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

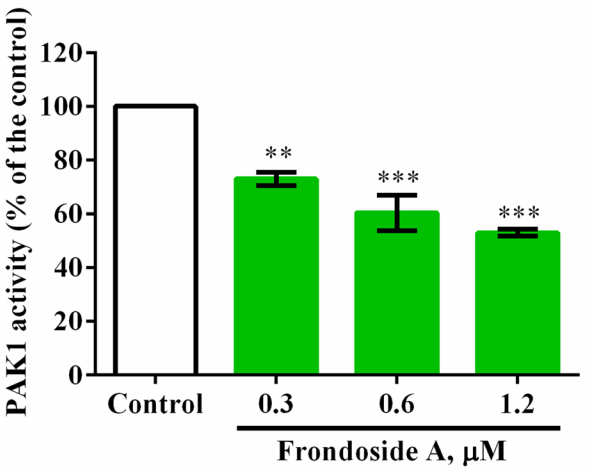

Figure 5. Frondoside A (FRA) directly inhibits PAK1 *in vitro*. PAK1 was treated *in vitro* with FRA at the indicated concentrations. The IC_{50} of FRA against PAK1 is around 1 µM. Asterisks on each bar indicate significant differences between treatment and control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Table 1. Anti-cancer activity and anti-kinase specificity of frondoside A and Okinawa propolis (OP) ingredients (nymphaeols)

Items	Anti-cancer (IC_{50})	Anti-PAK1 (IC_{50})	Anti-LIMK (IC_{50})	Anti-AKT (IC_{50})
Frondoside A	0.6	1.2	60	59
Okinawa propolis	12	10	39	30
Nymphaeol A	3.6	9.6	161	42
Nymphaeol C	6.5	9.8	171	74
Curcumin	23	16	30	44

IC_{50} value is in µM, except for Okinawa propolis (OP) in µg/mL.

around 60 μM (see Table 1), clearly indicating that FRA is indeed a potent PAK1-inhibitor, and its PAK1-specificity is even far more profound than that of nymphaeols.

To the best of our knowledge, OP is the very first propolis that has been proven to directly inhibit PAK1. All other propolis products in market such as ARC-based GP and CAPE-based Bio 30 block PAK1 only indirectly (by down-regulating RAC or other activators of PAK1).

Back in 2003, we found a rare potent marine poison called ST-2001, a 3-OH derivative of Staurosporine (ST), which directly inhibits PAK1, PKC and several other kinases with IC_{50} around 1 nM (*13*), but its anti-kinase mode of action is clearly "non-specific". Thus, to the best of our knowledge, FRA is the very first PAK1-specific inhibitor of marine origin. Currently, we are testing if FRA also could extend the healthy lifespan of *C. elegans*, as does OP (*7*).

Regarding the structure-function relationship of nymphaeols (see Figure 1 for chemical structures), either the position of geranyl side chain in nymphaeols or an extra short side chain in nymphaeol C does not seem to affect either their anti-PAK1 activity or kinase-specificity. In an attempt to determine the specific role of geranyl side chains in either anti-cancer/cell-permeability or anti-PAK1 activity/kinase specificity if any, we are planning to study the potential anti-cancer and anti-PAK1 activity of far simpler flavonoids such as naringenin and sakuranetin which contain no geranyl side chain.

Acknowledgements

We are very grateful to Mr. Md Shahinozzaman for his technical assistance to confirm the IC_{50} of Frondoside A against A549 cancer cell growth. This study was supported in part by the fishing company "Jinsho", Japan.

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(Received February 13, 2017; Revised April 6, 2017; Accepted April 9, 2017)