Sichuan Pepper Extracts Block the PAK1/Cyclin D1 Pathway and the Growth of NF1-Deficient Cancer Xenograft in Mice

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ABSTRACT

There is increasing evidence that more than 70% of cancers including pancreatic, breast and prostate cancers as well as neurofibromatosis (NF) are highly addicted to abnormal activation of the Ser/Thr kinase PAK1 for their growth. So far FK228 is the most potent among the HDAC (histone deacetylase) inhibitors that block the activation of both PAK1 and another kinase AKT, downstream of PI3 kinase. However, FK228 is still in clinical trials (phase 2) for a variety of cancers (but not for NF as yet), and not available for most cancer/NF patients. Thus, we have been exploring an alternative which is already in the market, and therefore immediately useful for the treatment of those desperate cancer/NF patients. Here we provide the first evidence that extracts of Chinese/Japanese peppercorns (Zanthoxyli Fructus) from the plant Zanthoxyllum piperitum called “Hua Jiao” /”Sansho”, block selectively the key kinase PAK1, leading to the downregulation of cyclin D1. Unlike FK228, these extracts do not inhibit AKT activation at the concentrations that block either cancer growth or PAK1 activation. The Chinese pepper extract selectively inhibits the growth of NF1-deficient malignant peripheral nerve sheath tumor (MPNST) cells, without affecting the growth of normal fibroblasts, and suppresses the growth of NF1-deficient human breast cancer (MDA-MB-231) xenograft in mice. Our data suggest that these peppercorn extracts would be potentially useful for the treatment of PAK1-dependent NF such as MPNST, in addition to a variety of PAK1-dependent cancers including breast cancers.

INTRODUCTION

Neurofibromatosis (NF), types 1 and 2 (NF1 and NF2), are disorders predisposing patients to tumors in their nerves, from which more than 1.5 million people on this planet are suffering.¹,² So far there is no effective therapeutic available for the treatment of NF. It is caused by loss-of-function mutations of either NF1 or NF2 tumor suppressor genes.¹,² NF1 gene product has been long known a RAS GAP (attenuator), and NF2 gene product called Merlin has recently been identified an inhibitor of PAK1, the key Ser/Thr kinase downstream of RAS.³ Thus, inactivation of either NF1 or NF2 causes abnormal activation of PAK1, and indeed the malignant growth of both NF1 and NF2 tumors was proven to require PAK1 as RAS transformation.³-⁵

Subsequently, at least two types of anti-PAK1 drugs have been found to suppress effectively neurofibroma xenografts in mice: (1) the most potent histone deacetylase (HDAC) inhibitor FK228⁵,⁶ and (2) a unique combination of two Tyr-kinase inhibitors, PP1 and GL-2003.⁷-¹⁰ However, none of these drugs has been available to NF patients as yet: FK228 is still at phase 2 of clinical trials only for cancers (excluding NF), while the PP1/GL-2003 combination has not entered even the phase 1 of clinical trials. In an attempt to find an immediate benefit for NF patients, we have started exploring alternative therapeutics available in the market, which would be potentially useful for the treatment of NF patients. There are several HDAC inhibitors/downregulators in the market, including the anti-epileptic drugs valproate (VPA), carbamazepine (CBZ), and curcumin (a yellow spicy ingredient of Indian curry),¹¹-¹³ which eventually block PAK1 activation. However, their IC50 for HDAC is rather high (above 1 µM), and these anti-epileptic drugs cause a few side effects such as rash, sleepiness, dizziness, and loss of short memory. Thus, a more potent and safer (with less side effect) compound that blocks PAK1 activation would be desirable for the effective signal therapy of NF.

Natural red/purple dyes (anthocyanins/anthocyanidins or other polyphenols) from a variety of vegetables such as bilberry, grapes, and purple corn have been recently reported to have an anti-cancer activity in cell culture or animal models.¹⁴,¹⁵ Some of anthocyanidins (aglycons) have been found to block EGF receptor/ErbB1-dependent pathways.¹⁶

KEY WORDS

Pepper, PAK1, Cyclin D1, NF1, MPNST, Cancer

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However, due to the high IC50 of these compounds (above 25 µM), it is unlikely that they are practical for the treatment of either cancers or NF. Resveratrol (RSVL) or its metabolite (piceatannol), dimethylallyl derivative RSVL-2 or methoxy derivatives such as MR3 and MR4 block the Tyr-kinase Syk, upstream of PI-3 kinase, leading to the inactivation of both PAK1 and another Ser/Thr kinase AKT.17-19 Again the IC50 of the natural polyphenol RSVL for both normal and cancer cell growth is far above 1 µM, although the synthetic derivatives MR3 and MR4 are much more potent (IC50 around 100 nM), and their toxicity is more selective towards cancer cells, with little effect on normal cell growth.18,19

The major spicy water-insoluble ingredient called “Sanshoohl” of Chinese/Japanese peppers (Zanthoxyl Fructis) has been used for more than two thousand years in East Asia as seasonings or traditional medicines for stimulating digestion or removing intestinal worms/parasites. In this brief report, we provide the first evidence that the water/ethanol-soluble red/brownish extracts of these peppers selectively block the PAK1/cyclin D1 pathway, and suppress the growth of MPNST cells in vitro and NF1-deficient (multidrug-resistant) cancer xenograft in mice.

**MATERIALS AND METHODS**

Preparation of extracts from Chinese/Japanese peppercorns (Zanthoxyl Fructis) The optimal conditions for the extraction of anti-PAK1 activity from Chinese peppercorns (Hua Jiao) purchased from a local grocery shop in Melbourne (or Hamburg) were soaked in 400 ml of 70% ethanol in water (v/v). The extraction was performed at 25°C by constantly rotating a 500 ml plastic container for several hours. The peppercorns were removed by filtering through a very fine stainless steel mesh for tea extraction, and the filtrate was then centrifuged at 2000 rpm for 5 min to remove the remaining insoluble powders. This clear extract (around 250 ml) is called “Kasui” (meaning a water-soluble extract of “Kasho” in Japanese) as the anti-PAK1 ingredient(s) is soluble in a warm (around 45°C) water as well. This extract contains 28 mg of dry materials per ml (measured after drying by a speed-vac at 50°C). The optimal conditions for the extraction of anti-PAK1 activity from Japanese pepper (Sansho) are: 5 g of the peppercorns (ground powder) from Wakayama Prefecture, Japan, purchased from a local grocery shop in Tokyo were suspended in 50 ml of 1% HCl in methanol (v/v), and the extraction was performed at 45°C in a water bath by occasional shaking for one hour. The mixture was then centrifuged at 2000 rpm for 5 min to obtain the clear supernatant (extract). This extract is called “Sansui” (meaning a water-soluble extract of “Sansho” in Japanese), again as the anti-PAK1 ingredient(s) is soluble in a warm (around 45°C) water as well. This extract (around 40 ml) contains around 25 mg of dry material per ml. The extract was then lyophilized to remove both HCl and methanol, and reextracted with 10 ml of 70% ethanol in water (v/v) at room temperature. The latter extract contains 5 mg of dry materials per ml. Both extracts were stored for a week in a refrigerator, and then kept frozen at minus 20°C.

**Cells lines.** The human NF1-deficient breast cancer cell line MDA-MB-231 was obtained from ATCC. Another NF1-deficient cell line S-462 was derived from a human malignant peripheral nerve sheath tumor (MPNST) of a NF1 patient, and is closely related to the NF1-deficient MPNST cell line S-805 described previously. In these MPNST cell lines both NF1 alleles are inactivated. The normal fibroblast cell line (FB1144.1) was derived from a healthy (non-NF) person who was operated for maxillofacial surgery.

Analysis of anti-mitotic activity of pepper extracts in vitro. 10^8 cells of MDA-MB-231 were seeded in a 96-well plate and incubated in 200 μl of culture medium RPMI-1640 containing 10% fetal calf serum (FCS) in the presence of a variety of concentrations of Chinese/Japanese pepper extracts for eight days, and cells were trypsinized and counted by a hemocytometer periodically. Each point (both time and concentration) was analyzed in triplicates, and the average was calculated. The standard deviation was less than 5%. 2 x 10^9 cells of either NF1-deficient MPNST cell line S-462 or the normal fibroblasts were seeded in a 96-well plate and cultured in the DME (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 2 mM Glu in the presence of a variety of concentrations of Chinese pepper extract for three days. Their growth was measured using the Cell proliferation kit II XTT (Roche, Basel, Switzerland), according to the manufacturer’s instruction. Briefly, tetratozolium salt (XTT) was added to the culture medium, where the viable cells reduce XTT through mitochondrial dehydrogenase to a purple formazan product, which is measured spectrophotometrically at 450 nm.20 The OD590 obtained for the nontreated cells was taken as 100% and all other values were normalized accordingly. Each point was repeated in four wells, and the average was calculated.

**Kinase assay and immunoblot of PAK1, AKT and cyclin D1 in cancer cells.** The MDA-MB-231 cells were treated with 70% ethanol extracts of Chinese or Japanese pepper (the final concentrations, 20 and 100 µg/ml, respectively) for 48 hours, and then cell lysates containing 1 mg of proteins (measured by Bradford assay) were subjected to PAK1 kinase assay and immuno-blotting with antibodies against PAK1, AKT, phospho AKT (Ser 473) and cyclin D1 as described previously.6,22

**Xenograft experiments.** BALB/c nu/nu mice (female, seven week old) were purchased from Animal Resources Center (Western Australia). MDA-MB-231 (5 x 10^6 cells per mouse) in 0.1 ml of a culture medium containing 50% Matrigel (BD Biosciences, Bedford, MA) were injected subcutaneously into two groups of eight nude mice. Twelve days later each group was treated intra-peritoneally with the 70% ethanol extract of Chinese pepper (110 mg/kg) or vehicle alone (0.4 ml of 5% DMSO in PBS) as the control, twice a week for 2~3 weeks. Under these conditions no mice suffered from any detectable adverse effect from these extracts. It should also be noted that, in accordance with Australia’s strict regulation of animal experimentation, all mice carrying solid tumors larger than 1 cm in diameter were immediately terminated for the animal welfare. Palpable tumor diameters were measured twice a week. Tumor volumes were calculated as:

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V = \frac{\pi}{4} \times [\text{length (mm)} + \text{width (mm)}]^2 \times \text{height (mm)}.
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**Statistical analysis.** Data are expressed as means ± SD (standard deviation), unless otherwise stated. Statistic analysis was performed mainly by means of Student’s t-Test. Differences were considered significant at p < 0.05.
RESULTS AND DISCUSSION

*Zanthoxyl fructus* extracts selectively inhibit the growth of human NF1-deficient cancer cells. The Chinese pepper extract strongly inhibits the growth of several human cancer cell lines including the NF1-deficient breast cancer cell line MDA-MB-231 where normal RAS is abnormally activated, due to no expression of NF1 gene encoding a RAS GAP, and the IC50 is around 10 µg/ml (see Fig. 1).

To test further the selectivity of the Chinese pepper extract’s effects, its anti-mitotic activity was compared between NF1-deficient malignant peripheral nerve sheath tumor (MPNST) cells and normal fibroblasts of human origin. As shown in Figure 2, the pepper extract at 20 µg/ml has little effect on the growth of normal fibroblasts, while it inhibits the growth of MPNST by around 90%. The results clearly suggest that its effect is highly specific on the NF1-deficient cancer cells in which both RAS and PAK1 are abnormally activated.5,10

At the concentrations higher than 320 µg/ml, the extract causes the cell death (apoptosis), suggesting that one of its targets might be the PI-3 kinase pathway, which is essential for cell survival.

Chinese/Japanese pepper extracts selectively block PAK1 activation and cyclin D1 upregulation in cancer cells. In order to understand the molecular mechanism underlying the anti-mitotic activity of these extracts, we examined their effects on both kinases PAK1 and AKT which act downstream of PI-3 kinase. As shown in (Fig. 3), both Chinese and Japanese pepper extracts around the IC50 for cell growth (0.02 mg/ml and 0.1 mg/ml, respectively) selectively block the activation of PAK1, without affecting either its protein level, AKT protein levels or activation of AKT (phospho AKT levels). PAK1 is essential for RAS-induced upregulation of cyclin D1.22 As predicted, both extracts strongly block cyclin D1 upregulation (see Fig. 3), which is essential for the activation of cyclin-dependent kinases (CDKs), leading to the G1 to S transition of cell cycle for the initiation of DNA replication (see Fig. 4).

Chinese pepper extract suppresses the growth of NF1-deficient cancer xenograft in mice. The human cancél cell line MDA-MB-231 has mainly three unique properties. It does not express NF1, a RAS GAP, and therefore its tumor can serve as a model of the type 1 neurofibromatosis in vivo. Furthermore, although it was derived from a breast cancer, its growth is independent of estrogen, and it is resistant to a variety of anti-cancer drugs including FK228.6 We have examined whether the Chinese pepper extract has any therapeutic effect on this NF1-deficient experimental NF tumor in vivo. This extract (110 mg/kg, i.p., twice a week) suppressed almost completely the growth of this NF1-deficient tumor xenograft in mice at least during the first two weeks treatment (see Fig. 5), without any detectable adverse effect on the treated animals. However, thereafter the tumor started to grow slowly even with this herbal "Kasui" extract treatment. The result suggests at least two possibilities: (1) a “Kasui”-resistant mutant population of this cell line began taking over the whole tumor population, or (2) this extract upregulates a multi-drug resistant (MDR) protein in the cells that would pump the anti-PAK1 ingredient out. A similar result was obtained independently, by another group with the warm water-soluble extract of Japanese pepper, testing on tumor xenografts derived from MDA-MB-231 and a few other cancer cell lines, but at apparently higher doses.23

These results suggest that the Chinese/Japanese pepper extracts would be among the first effective NF therapeutics available in the market. However, to handle the emerging drug-resistance problem, we are currently examining whether another herbal product called diindolylmethane (DIM), the major anti-cancer metabolite of indole 3 carbinol (I3C) derived from broccoli and other cruciferous vegetables, which blocks RAS-induced oncogenic/angiogenic signaling pathway involving both AKT and PAK1 by upregulating the PIP3 phosphatase PTEN,24-27 or any other PAK1 blockers such as GL-2003, can overcome such a “Kasui”-resistance of this tumor. Interestingly, when used together GL-2003 and another Tyr-kinase inhibitor called PP1, both of which eventually block RAS-induced PAK1 activation, appear to overcome the resistance to each drug of tumors derived from both this cell line and human pancreatic cancer cell line.10 Also both purification and chemical identification of the water/ethanol-soluble responsible (anti-PAK1) ingredient in the
red-brown Chinese/Japanese pepper extracts are under way, in an attempt to separate first the anti-PAK1 ingredient(s) from the rest in this extract. So far no water-soluble natural compound has ever been identified that selectively blocks PAK1 activation. Thus, it is likely that the anti-mitotic component in these extracts would be a new compound(s).

Regarding an orthotopic animal model of NF1 and NF2, very recently Breakefield’s group is reportedly successful in demonstrating the first effective therapeutic effect of herpes simplex virus (HSV) on Schwannomas developed in NF2 transgenic mouse.28 Thus, we are planning to collaborate with this group to test the therapeutic effect of Chinese peppercorn extract, DMI, or their combination on this NF2 mouse model. Although an NF1 transgenic mouse model was also developed several years ago,29 so far nobody has published any successful therapeutic effect on this mouse model, which is reportedly good for leukemia, but not suitable for solid tumors such as neurofibromas and Schwannomas.

References