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CAPE (Caffeic Acid Phenethyl Ester)-based Propolis Extract (Bio 30) Suppresses the Growth of Human Neurofibromatosis (NF) Tumor Xenografts in Mice

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Dysfunction of the *NF1* gene coding a RAS GAP is the major cause of neurofibromatosis type 1 (NF1), whereas neurofibromatosis type 2 (NF2) is caused primarily by dysfunction of the *NF2* gene product called merlin that inhibits directly PAK1, an oncogenic Rac/CDC42-dependent Ser/Thr kinase. It was demonstrated previously that PAK1 is essential for the growth of both NF1 and NF2 tumors. Thus, several anti-PAK1 drugs, including FK228 and CEP-1347, are being developed for the treatment of NF tumors. However, so far no effective NF therapeutic is available on the market. Since propolis, a very safe healthcare product from bee hives, contains anticancer ingredients called CAPE (caffeic acid phenethyl ester) or ARC (artepillin C), depending on the source, both of which block the oncogenic PAK1 signaling pathways, its potential therapeutic effect on NF tumors was explored *in vivo*. Here it is demonstrated that Bio 30, a CAPE-rich water-miscible extract of New Zealand (NZ) propolis suppressed completely the growth of a human NF1 cancer called MPNST (malignant peripheral nerve sheath tumor) and caused an almost complete regression of human NF2 tumor (Schwannoma), both grafted in nude mice. Although CAPE alone has never been used clinically, due to its poor bioavailability/water-solubility, Bio 30 contains plenty of lipids which solubilize CAPE, and also includes several other anticancer ingredients that seem to act synergistically with CAPE. Thus, it would be worth testing clinically to see if Bio 30 and other CAPE-rich propolis are useful for the treatment of NF patients. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: propolis; CAPE; neurofibromatosis; NF1; NF2; Bio 30; MM; AIDS; Fragile X syndrome.

INTRODUCTION

Propolis from bee hives (also called 'bee wax') contains a 100 million years of wisdom of bee colonies protecting their larva from a variety of diseases. Propolis has been used since ancient Egypt times as a traditional/folk medicine for the treatment of infection, wound and inflammation as well as for preparing mummies. Entering our modern era, propolis was first identified as an anticancer remedy in the late 1980s when Dezider Grunberger's group at Columbia University found that CAPE (caffeic acid phenethyl ester) was the major anticancer ingredient in a propolis sample from Israel (Grunberger *et al.*, 1988). CAPE is a derivative of caffeic acid (CA) that down-regulates the GTPase Rac, a direct activator of the kinase PAK1 (Xu *et al.*, 2005). As a consequence, CAPE eventually inactivates PAK1. Interestingly, a propolis from New Zealand (NZ) reportedly showed the highest CAPE content (6–7% of extract) of a variety of propolis samples from around the world, whereas Brazilian green propolis contains another anticancer ingredient called ARC (artepillin C), instead of CAPE, which also inactivates PAK1 (Maruta and Ohta, 2008).

Recently we and others have shown that more than 70% of human cancers and all NF (neurofibromatosis) tumors are highly dependent on PAK1 for their growth, but not normal cells (Sasakawa *et al.*, 2003; Hirokawa *et al.*, 2004, 2005a, 2005b; Maruta and Ohta, 2008). Dysfunction of the *NF1* gene coding a RAS GAP is the major cause of type 1 NF, whereas type 2 NF is caused primarily by dysfunction of the *NF2* gene product called merlin that inhibits directly PAK1, a Rac/CDC42-dependent Ser/Thr kinase (Hirokawa *et al.*, 2004). Several years ago, *NF1*-deficient tumors were shown to require PAK1 for their growth *in vivo*, and more recently it was found that *NF2*-deficient tumors also require the same kinase for their growth *in vitro* (Hirokawa *et al.*, 2004). However, so far no effective NF therapeutic is available on the market. Thus, we have been developing a series of anti-PAK1 drugs which would be useful for the treatment of these PAK1-dependent cancers or tumors. Of the anti-PAK1 drugs/ingredients that have been identified or developed for the treatment of NF1

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and NF2 tumors, a natural ring peptide FK228 appears to be the most potent both *in vitro* and *in vivo*. The IC_{50} for NF tumor cells is around 5 pM *in vitro*, and causes the complete regression of *NF1*-deficient MPNST xenograft in mice (Hirokawa *et al.*, 2005b). Its direct target is an HDAC (histone deacetylase), and it activates a specific set of tumor suppressor genes such as p21 (a CDK inhibitor) and RAP1a (a RAS antagonist), which eventually block both up-stream and down-stream of PAK1 (Hirokawa *et al.*, 2005a). Unfortunately, FK228 is still in clinical trials (phase 2) for cancers, but not for NF, and it would take several more years for this powerful drug to enter NF trials, and eventually to give any benefit to NF patients.

So we have recently started developing anti-PAK1 ingredients from natural products which are inexpensively available on the market, hoping to give the immediate benefit to NF patients. The first natural anti-PAK1 ingredient(s) was found in an ethanol extract of Chinese (Sichuan) pepper which selectively blocks PAK1 activation, but not another kinase called AKT (Hirokawa *et al.*, 2006). This extract suppresses the growth of *NF1*-deficient cancer xenografts in mice by 50%. More recently we found there was a much more potent natural anti-NF therapeutic called 'Bio 30'. It is a water-miscible CAPE-rich extract of NZ (New Zealand) propolis. This study demonstrates that Bio 30 suppressed completely the growth of both NF1 and NF2 tumor xenografts in mice, suggesting the possibility that Bio 30 and other CAPE-rich propolis might serve as the first effective and very safe NF therapeutic inexpensively available on the market.

MATERIALS AND METHODS

Tumor xenografts in mice. 6-week-old female nu/nu mice (from Charles River or NCI) received 5 million MPNST (S-462) cells (Hirokawa *et al.*, 2005b, 2006) or 2–5 million *NF2*-deficient Schwannoma (HEI-193) cells (Prabhakar *et al.*, 2007; Lepont *et al.*, 2008) of human origin in 0.2 mL of 50% Matrigel per mouse subcutaneously in the flank or thigh. When the average size of the tumors reached around 5 mm diameter (length or width), groups of 5–7 mice were treated with either Bio 30 (100–300 mg/kg) alone or CAPE 60 (Bio 30 plus extra 5 mg/kg CAPE) i.p., twice a week, while the control mice were treated with the vehicle (11% propylene glycol and 26% DMSO in PBS). The size of each tumor (both the original and metastasized) was measured twice a week by calliper. None of these treatments caused any adverse effect on the mice.

Cell culture. 10^3 cells of MPNST or Schwannoma cell lines were seeded per well, and cultured for 3–6 days, in the presence or absence of Bio 30, CAPE or ARC at various concentrations and the growth was monitored by either directly counting viable cells with a hemocytometer or MTT method (measuring the optical density at 550 nm) as described previously (Mosmann, 1983; Hirokawa *et al.*, 2006).

Polyphenol content analysis of Bio 30. The Bio 30 (alcohol-free liquid) sample was extracted by ethyl acetate and subjected to liquid chromatography to analyse its major polyphenols including CAPE by the tandem mass spectrometric method described previously (Celli *et al.*, 2004).

RESULTS AND DISCUSSION

Effect of Bio 30 on the growth of NF1 and NF2 tumor cells *in vitro*

Since it was found that Bio 30 (Manuka Health, NZ) was more potent than the Chinese pepper extract (Hirokawa *et al.*, 2006) in inhibiting the growth of PAK1-dependent pancreatic and breast cancer cell lines both *in vitro* and *in vivo* (Y. Hirokawa and H. Maruta, unpublished observation), its effect on NF tumor cell lines was examined *in vitro* to begin with. The IC_{50} of Bio 30 for NF2 tumor (HEI-193) and NF1 cancer (MPNST/ S-462) cells turned out to be around 1.5 μg/mL and 8 μg/mL, respectively (see Fig. 1).

Effects of Bio 30 on the growth (or metastasis) of NF tumor xenografts in mice

Effect on the growth of *NF1*-deficient MPNST. Bio 30 (100 mg/kg, i.p., twice a week) suppressed completely

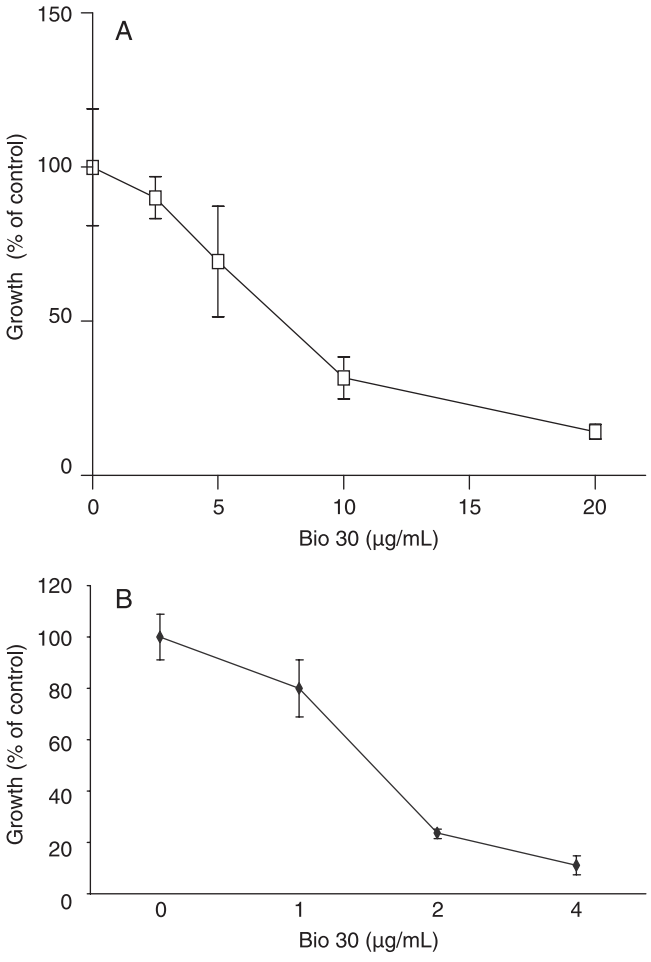

Figure 1. Bio 30 inhibits the growth of NF tumor cells MPNST (A) and Schwannoma (B) cells cultured for 6 days in the presence or absence of Bio 30 at the concentrations indicated as described under Materials and Methods.

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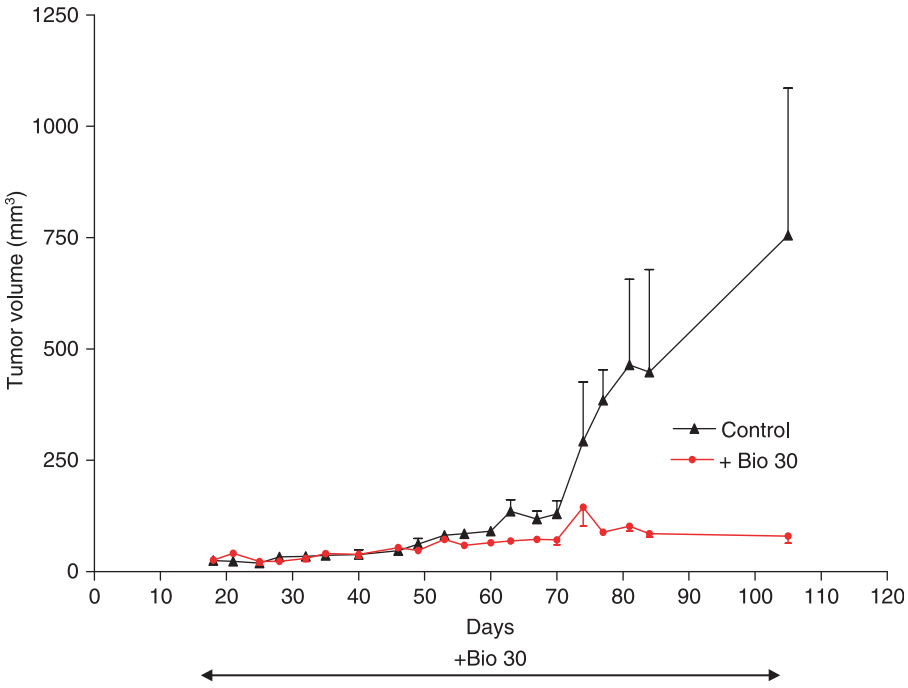

Figure 2. Bio 30 suppresses the growth of MPNST *in vivo* in nude mice bearing MPNST on the flank were treated with Bio 30 (100 mg/kg), i.p., twice a week during days 19–100, as described under Materials and Methods. Each group consisted of five mice.

the slow growth of MPNST /S-462 xenograft in mice over 100 days (see Fig. 2). Since this MPNST is poorly angiogenic, it remains dormant for the first 10 weeks until the blood vessel forms sufficiently around a tiny tumor (around 5–6 mm in a diameter). Then tumors in the control mice burst to growth. However, tumors in the Bio 30-treated mice failed to grow, probably because the CAPE-rich propolis blocked the angiogenesis, in addition to cell division and metastasis of the MPNST cells.

Effects on the highly metastatic *NF1*-deficient MPNST. It was found that the MPNST became highly metastatic and grew much more slowly if MPNST cells were inoculated in the thigh of a back leg, instead of the flank. This provided a good opportunity to examine the antimetastatic effect of Bio 30 (100 mg/kg) alone or its combination with an extra CAPE (5 mg/kg) called 'CAPE 60' (containing 60 mg of CAPE, instead of 12 mg of CAPE, per g of Bio 30 extract) on the MPNST. At around 6 weeks, the metastasis of tumors began in the control mice (Fig. 3). However, in the Bio 30 (alone)-treated mice, the total number of metastasized tumors was reduced significantly. Furthermore, extra CAPE significantly delayed the onset of metastasis and enhanced the antimetastatic effect of Bio 30. In addition, Bio 30 alone or its combination with extra CAPE (CAPE 60) caused the regression of MPNST: while all the control mice bore tumors, a third of Bio 30-treated mice and two thirds of CAPE 60-treated mice lost the tumors completely over 10 weeks. PAK1 is known to be essential for anchorage-independent growth, mestastasis

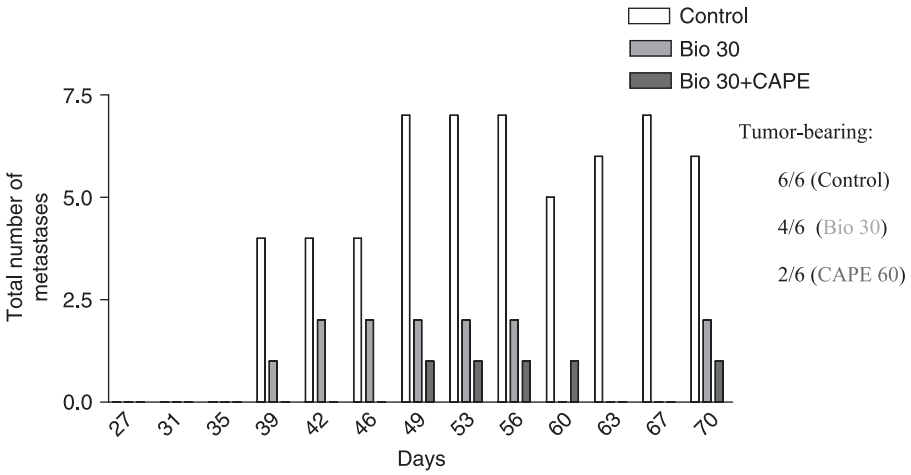

Figure 3. Bio 30 alone or its combination CAPE 60 blocks metastasis of MPNST in nude mice bearing MPNST on the thigh treated with either Bio 30 (100 mg/kg) alone or CAPE 60, i.p., twice a week during days 27–63, as described under Materials and Methods. Each group consisted of six mice. While all the control mice continued carrying tumors, the Bio 30 alone-treated mice lost two original tumors, and the CAPE-treated mice lost four original tumors by day 63. The number of metastasized tumors represents the total number of those in each group, and not the average per mouse of each group.

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and angiogenesis of cancers in general (Kiosses *et al.*, 2002; Maruta and Ohta, 2008). These observations together strongly suggest that CAPE is the limiting antitumor ingredient of Bio 30, and extra CAPE would boost both its antimetastatic and antimitotic (or/and antiangiogenic) effects on the MPNST. The richer in CAPE, the more therapeutic a propolis might be (on NF tumors).

Effect on the growth of *NF2*-deficient Schwannoma

Bio 30 alone (100 mg/kg, i.p., twice week) caused an almost complete regression of the fast-growing human *NF2*-deficient Schwannoma (HEI-193) xenograft in mice over 30 days (see Fig. 4A). This regression occurred only when the drug treatment started at an early stage of tumor growth (with the average volume of tumors being around 150 mm^3).

However, if the treatment started after the tumor size reached around 400 mm^3, the therapeutic effect of Bio 30 was hardly observed at this dose (data not shown). With the tripled dose (300 mg/kg), even if the drug treatment started when the tumor size reached over 600 mm^3, Bio 30 alone could suppress the growth of Schwannoma almost completely for 30 days (see Fig. 4B). This size

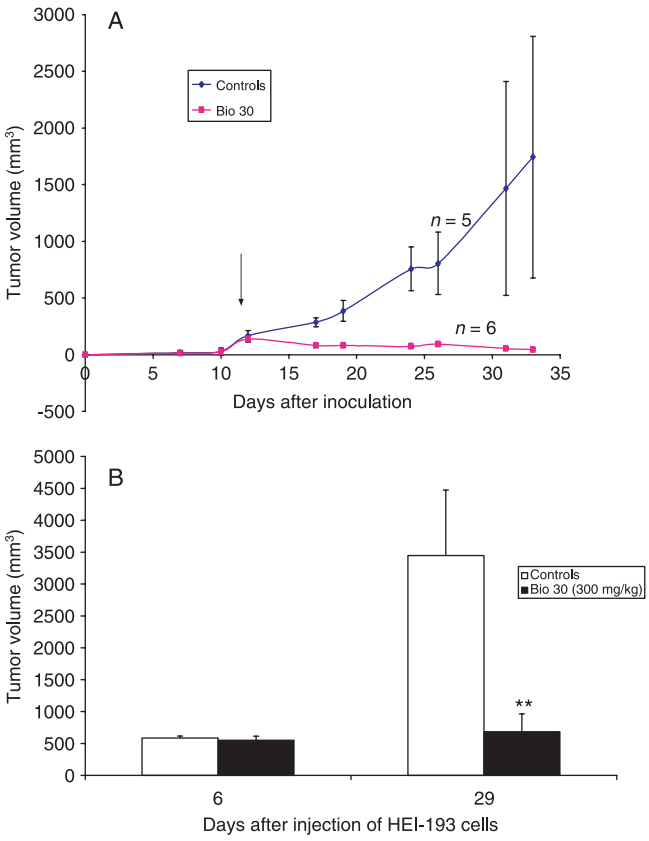

Figure 4. Bio 30 alone suppresses the growth of Schwannoma *in vivo* in nude mice bearing Schwannoma on the flank treated with (A) Bio 30 (100 mg/kg), during days 14–31, or (B) Bio 30 (300 mg/kg), during days 6–27, i.p., twice a week as described under Materials and Methods. (A) drug-treatment began when the average volume of tumors was around 150 mm^3, and (B) the treatment began when the tumor volume was just over 600 mm^3. Histogram (B): the control (blank, $n = 7$), and the Bio 30 treated (filled, $n = 7$).

Table 1. Major polyphenol content of Bio 30

Compound	mg/g
Pinocembrin	110
Galangin[a]	60
Chrysin[a]	30
CAPE[a]	12
CA[a]	12
Apigenin[a]	12

The ethyl acetate extract of Bio 30 was analysed as described under Materials and Methods.
The source of Bio 30 was Manuka Health, Auckland, New Zealand.
[a]Anticancer compounds.

of NF2 tumors in mice (weighing around 20 g) is equivalent to that of a fairly large NF2 tumor (over 1.5 kg) for people (weighing around 50 kg). These observations seem to parallel the clinical situation in that the earlier the treatment begins, the better the chance of complete tumor regression.

CAPE alone versus Bio 30

Propolis such as Bio 30 contains plenty of lipids which solubilize CAPE and a few other anticancer polyphenols such as galangin, chrysin and apigenin (see Table 1). Furthermore, CAPE appears to work synergistically with these other polyphenols in Bio 30 to suppress the growth of these NF tumor cells at least *in vitro*. For instance, as mentioned before, the IC_{50} of Bio 30 for the Schwannoma cell line is around 1.5 μg/mL. Since the CAPE content of this propolis extract is only 1.2% (12 mg/g of extract), the contribution of CAPE alone to 1.5 μg/mL of Bio 30 is only around 18 ng/mL, that is to say, around 60 nM. The IC_{50} of CAPE alone for the same cells is as high as about 36 μM (see Fig. 5) which

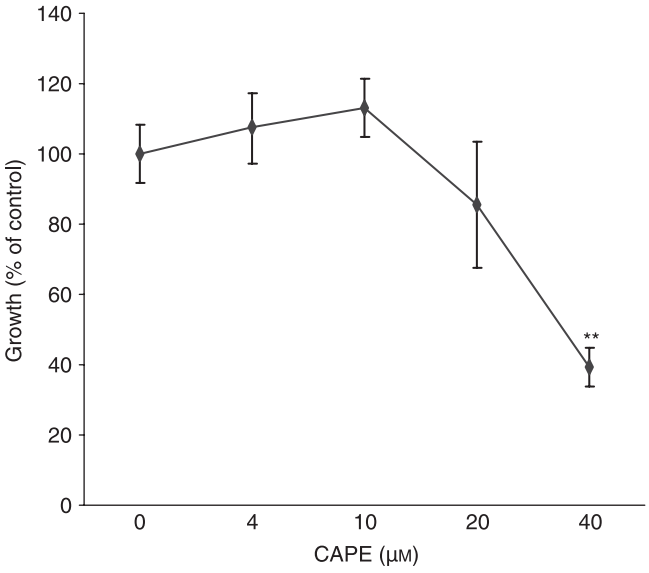

Figure 5. IC_{50} of CAPE alone for the growth of Schwannoma cells. Cells were cultured for 6 days in the presence or absence of CAPE at the indicated concentrations, and the cell growth was monitored as described in Fig. 1B.

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is around 600 times higher than 60 nM. Since the IC_{50} of a few other anticancer ingredients such as galangin, chrysin and apigenin is known to be around a similar range (10–40 μM) for various cancer cells (Murray *et al.*, 2006; Maruta and Ohta, 2008), it is most likely that CAPE acts synergistically (rather than simply additively) with these anticancer ingredients in Bio 30. In other words, the water-miscible Bio 30 would be far more effective than the water-insoluble CAPE alone for the treatment of NF tumors. Besides propolis in general is known to have an additional (indirect) anticancer function that boosts the immune system, in particular the lytic action of natural killers against tumor cells, and the antibody production (Sforcin, 2007).

Bio 30 trials

Since Bio 30 is a very safe healthcare food supplement, a trial is being conducted of Bio 30 (daily oral treatment with a dose of 25 mg/kg) for, so far, around 70 patients of body weight over 25 kg (or over 10 years old) who are suffering from either NF or formidable cancers such as melanoma and pancreatic cancers in which RAS is mutated and therefore PAK1 is abnormally activated. Its daily cost per person (adult) is only a dollar. The only minor potential problem with CAPE-based propolis such as Bio 30 is that less than 5% of people are allergic to CAPE. In our Bio 30 trial, so far only one person has shown an allergic skin rash, and switched to ARC-based Brazilian green propolis which does not cause any allergy but costs around 10 times as much as Bio 30.

Although our trial is still at a very early stage (less than 12 months for NF1 patients and 6 months for NF2 and a few other cancer patients), so far the majority of these patients showed a positive outcome from Bio 30, namely no further growth of their tumors.

It is our hope that this work will set the stage, a milestone for much more sophisticated and comprehensive clinical studies in the future for testing the effect of Bio-30 not only on the growth of NF tumors and PAK1-dependent formidable cancers such as pancreatic cancers, melanomas and MM (multiple myeloma), but also several other PAK1-dependent diseases such as AIDS (HIV-infection) and Fragile X mental retardation syndrome (Nguyen *et al.*, 2006; Hayashi *et al.*, 2007; Porchia *et al.*, 2007; Zhang *et al.*, 2007; Maruta and Ohta, 2008).

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