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Hypoxia affects cellular responses to plant extracts

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ABSTRACT

Ethnopharmacological relevance: Microenvironmental conditions contribute towards varying cellular responses to plant extract treatments. Hypoxic cancer cells are known to be resistant to radio- and chemo-therapy. New therapeutic strategies specifically targeting these cells are needed. Plant extracts used in Traditional Chinese Medicine (TCM) can offer promising candidates. Despite their widespread usage, information on their effects in hypoxic conditions is still lacking. In this study, we examined the cytotoxicity of a series of known TCM plant extracts under normoxic versus hypoxic conditions.

Materials and methods: Pereskia grandifolia, Orthosiphon aristatus, Melastoma malabathricum, Carica papaya, Strobilanthes crispus, Gynura procumbens, Hydrocotyle sibthorpioides, Pereskia bleo and *Clinacanthus nutans* leaves were dried, blended into powder form, extracted in methanol and evaporated to produce crude extracts. Human Saos-2 osteosarcoma cells were treated with various concentrations of the plant extracts under normoxia or hypoxia (0.5% oxygen). 24 h after treatment, an MTT assay was performed and the IC_{50} values were calculated. Effect of the extracts on hypoxia inducible factor (HIF) activity was evaluated using a hypoxia-driven firefly luciferase reporter assay.

Results: The relative cytotoxicity of each plant extract on Saos-2 cells was different in hypoxic versus normoxic conditions. Hypoxia increased the IC_{50} values for *Pereskia grandifola* and *Orthosiphon aristatus* extracts, but decreased the IC_{50} values for *Melastoma malabathricum* and *Carica papaya* extracts. Extracts of *Strobilanthes crispus, Gynura procumbens, Hydrocotyle sibthorpioides* had equivalent cytotoxic effects under both conditions. *Pereskia bleo* and *Clinacanthus nutans* extracts were not toxic to cells within the concentration ranges tested. The most interesting result was noted for the *Carica papaya* extract, where its IC_{50} in hypoxia was reduced by 3-fold when compared to the normoxic condition. This reduction was found to be associated with HIF inhibition.

Conclusion: Hypoxia variably alters the cytotoxic effects of TCM plant extracts on cancer cells. *Carica papaya* showed enhanced cytotoxic effect on hypoxic cancer cells by inhibiting HIF activities. These findings provide a plausible approach to killing hypoxic cancer cells in solid tumors.

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1. Introduction

The use of plant extracts in Traditional Chinese Medicine (TCM) can be traced back to 6000 years ago (Solecki and Shanidar, 1975). In recent years, there has been a burgeoning use of plant extracts in TCM for modern drug discovery (Graziose et al., 2010). These plant extracts are used for a variety of purposes including health improvement, beauty, and nutritional supplementation, as well as prevention and treatment of diseases, including diabetes and cancer (Graziose et al., 2010).

Oxygen homeostasis is a critical element for physiological well being of the human body. Limited oxygen, termed hypoxia, plays a major role in the pathobiology of solid tumors. Cells in hypoxic regions of tumors are more resistant to radiation and chemotherapy (Brown, 2000). Master regulators of cell survival under hypoxia are the hypoxia-inducible factors (HIFs), HIF-1 and HIF-2. These transcription factors regulate several processes vital for the cells to survive the hypoxic conditions (Semenza, 2011). Since these cancer cells have altered metabolic mechanisms for survival under hypoxia, it is conceivable that their responses to plant extracts also will be different from normoxic cancer cells. Therefore, identification of new agents to kill cancer cells in hypoxic areas is crucial. In the present study, we investigated the cytotoxic effects of selected plant extracts, with known medicinal properties (Supplemental

Abbreviations: TCM, Traditional Chinese Medicine; HIF, Hypoxia-inducible factor

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Table 1), on cancer cells in culture, under normoxic and hypoxic conditions.

2. Materials and methods

2.1. Cell line and culture conditions

Cultured Saos-2 human osteosarcoma cells were used in the study. A normoxic or hypoxic environment was created by incubating cells at 37 °C either in a humidified CO_2 incubator or a ProOx in vitro chamber (BioSpherix) supplied with 0.5% O_2, respectively (Kaluz et al., 2008).

2.2. Plant materials, cell culture treatment and viability assay

Nine types of plants with known medicinal properties (Supplemental Table 1) were tested in this study. The voucher specimens were deposited in the Herbarium of Institute of Biosciences, Universiti Putra Malaysia. Leaves of these plants were subjected to methanolic extraction as described in Hsu et al., 2010. Prior to use, the extracts were dissolved in serum-free media with a final DMSO concentration of less than 0.5% (v/v), centrifuged at 1 000 xg and filtered through 0.22 μM filters. Overnight cultures of cells, initially seeded at 1.5×10^4 cells per well in a 96-well plate, were treated with selected concentrations of plant extracts and incubated in normoxic or hypoxic conditions. After 24 h of incubation, an MTT assay (Jamal et al., 2012) was performed.

2.3. HIF reporter assay

Cells were co-transfected with a hypoxia-driven firefly luciferase reporter plasmid construct containing four copies of the erythropoietin (EPO) hypoxia response elements (HRE) and a pRL-CMV expressing *Renilla* luciferase as described previously (Kaluz et al., 2008, Shafee et al., 2009). Transfected cells were treated with appropriate concentrations of plant extracts and incubated in either normoxic or hypoxic conditions. After 24 h, firefly and *Renilla* luciferase signal intensities were measured (Kaluz et al., 2008, Shafee et al., 2009). Percent HIF inhibition was calculated as a ratio of the difference between untreated and plant extract-treated sample to the untreated sample. A positive value indicates HIF inhibition, while a negative value denotes HIF activation.

2.4. Statistical analysis

The Student t-test was used to analyze the experimental data in this study. Results were expressed as mean ± standard error of the mean (SEM). A p value of < 0.05 was considered significant.

3. Results and discussion

To investigate whether hypoxia affects cellular responses to plant extract treatment, cells were treated with selected concentrations of each extract, and their viability was determined. As positive controls for inhibition of HIF activity, we included cisplatin (Duyndam et al., 2007), and Chetomin (Tan et al., 2005). Different patterns of cytotoxicity were observed when the cells were treated in normoxic versus hypoxic conditions. As expected (Song et al., 2006), the IC_{50} value for cisplatin in hypoxia is significantly higher than in normoxia (Fig. 1A). For the first time, we show that the IC_{50} of chetomin also increased in hypoxia.

Melastoma malabathricum, *Strobilanthes crispus* and *Pereskia grandifolia*, showed no drastic differences in cytotoxicity when lower concentrations (< 50 μg/ml) of extracts were used (Fig. 1A). However, at concentrations higher than 150 μg/ml, cytotoxicity to *Melastoma malabathricum* became more evident in the hypoxic compared to the normoxic cultures. Cytotoxic and antiproliferative activities of methanolic extracts of *Melastoma malabathricum* have been previously reported (Devehat et al., 2002). Their studies, which were done under normoxia, showed that the IC_{50} values of the extract ranges from 19 to > 400 μg/ml depending on the cell line tested. Results in the present study showed that hypoxic cancer cells are more susceptible to *Melastoma malabathricum* cytotoxicity when the concentrations used are above 150 μg/ml. Therefore, it is likely that the IC_{50} of the extracts will be lower in hypoxic cancer cells. For *Strobilanthes crispus* the difference in cytotoxicity began to be seen as early as 100 ug/ml. Beginning at this concentration, cells in the hypoxic environment showed a higher rate of cell death compared to the cells in the normoxic environment. The opposite situation was observed in the cells treated with *Pereskia grandifolia*. Cytotoxicity of the cells was observed to be higher in the normoxic condition instead, even though cytotoxicity was seen earlier, at 25 μg/ml.

Gynura procumbens, Hydrocotyle sibthorpioides and *Carica papaya* extracts showed different cytotoxicities in normoxia and hypoxia at all concentrations used. *Gynura procumbent* and *Hydocotyle sibthorpioides* were found to induce cell proliferation at concentrations lower than 150 μg/ml. No induction was seen for *Carica papaya* extract. Interestingly, in hypoxia, all these extracts induced higher cell proliferation than in normoxia. To the best of our knowledge, this is the first report to show induction of cell proliferation in cancer cells by these three plant extracts. *Gynura procumbens* has been shown to contribute towards the wound healing process (Zahra et al., 2011). Our findings of increased cell proliferation, albeit in cancer cells, may help contribute towards further understanding of mechanisms involve in the process of wound healing. Similar to our findings in the normoxic condition, the inefficiency of *Hydrocotyle sibthorpioides* extracts in cell killing was also reported previously (Huang et al., 2008). They showed that the IC_{50} of *Hydrocotyle sibthorpioides* ethanolic extracts in cancer cells was > 2000 μg/mL. For *Carica papaya,* we observed a minimal stimulatory effect in hypoxia at concentrations lower than 250 μg/ml. However, above this concentration, its cytotoxicity increased tremendously. In normoxia, on the other hand, the extract showed a gradual increase in cytotoxicity with increasing concentrations of extracts. This result is supported by Otsuki et al. (2010) which reported anti-proliferative responses of various tumor cell lines in normoxic conditions towards *Carica papaya* extracts.

Orthosiphon aristatus, Pereskia bleo and *Clinacanthus nutans* extracts showed minimal cytotoxicity in both normoxic and hypoxic conditions. *Orthosiphon aristatus* extract, which was previously shown to have antioxidant and anti-inflammatory effects (Hsu et al., 2010), showed a gradual reduction of cell viability with increasing amounts of extract used in the normoxic condition. But in the hypoxic condition, no statistically significant difference in cytotoxicity was observed until 500 ug/ml were used, when a sharp drop in cell viability was observed in both normoxic and hypoxic conditions, indicating a general cytotoxity. For *Pereskia bleo* and *Clinacanthus nutans* extracts, no significant cytotoxicity was seen until at the highest concentrations tested under normoxic conditions. This finding is in agreement with a study by Er et al. (2007) which also failed to observe any notable anti-proliferative effect of *Pereskia bleo* methanolic extract in 4T1 and NIH/3T3 cell lines. In contrast, Malek et al. (2009) reported cytotoxicity effects of *Pereskia bleo* methanolic extract in several cancer cell lines. Besides the different types of cancer cells used, another possible explanation for these inconsistencies is the gaseous conditions used in their studies. In our study, we found that hypoxic environment led to growth stimulatory response by *Pereskia bleo* but not for *Clinacanthus nutans*. This result strongly suggests that microenvironmental conditions contribute towards cellular responses to plant extract treatments.

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