RESEARCH COMMUNICATION

A Synthetic Hydrazone Derivative Acts as an Apoptotic Inducer with Chemopreventive Activity on a Tongue Cancer Cell Line

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Abstract

One of the main aims of cancer chemopreventive studies is to identify ideal apoptotic inducers, especially examples which can induce early apoptotic activity. The present investigation focused on chemopreventive effects of a hydrazone derivative using an in vitro model with tongue cancer cells. Alteration in cell morphology was ascertained, along with stage in the cell cycle and proliferation, while living-dead status of the cells was confirmed under a confocal microscope. In addition, cytotoxicity test was performed using normal mouse skin fibroblast cells. The results showed that the compound inhibited the growth of tongue cancer cells with an inhibitory concentration (IC_{50}) of 0.01 mg/ml in a dose and time-dependent manner, with a two-fold increase in early apoptotic activity and G0G1 phase cell cycle arrest compared to untreated cells. Exposure to the compound also resulted in alterations of cell morphology including vacuolization and cellular shrinkage. Confocal microscope analysis using calcein and ethidium staining confirmed that the compound caused cell death, whereas no cytotoxic effects on normal mouse skin fibroblast cells were observed. In conclusion, the findings in this study suggested that the hydrazone derivative acts as an apoptotic inducer with anti-proliferative chemopreventive activity in tongue cancer cells.

Keywords: Chemoprevention - apoptosis - hydrazones - tongue cancer cells

Asian Pacific J Cancer Prev, **12**, 259-263

Introduction

Cancer is a leading cause of death throughout the world and Malaysia is of no exception. However, the conventional treatments available for cancer disease, such as chemotherapy or radical surgery, eventually fail to exert control on the disease. Metastatic disease frequently develops even after these treatments and may cause death.

Therefore, interest has focused on chemoprevention which aims at suppressing, delaying or reversing carcinogenesis by pharmacologic intervention with naturally occurring or synthetic agents (Sporn and Suh, 2002), (Tsao et al., 2004). Cancer chemoprevention has been an active area of research for several decades and the use of retinoids to prevent cancer of the head and neck is a notable example (Khuri et al., 1997). Despite this, to date, relatively little research has been done on oral cancer chemoprevention.

Scientists from School of Chemical Sciences, Universiti Sains Malaysia (USM) have synthesized a number of new organic compounds in the class of compounds known as the hydrazones. This is an important class of compounds for new drug development and some of the hydrazone derivatives have shown to exhibit anti-cancer properties (Rollas and Kucukguzel, 2007). For example, (2,6-dimethyl-N'-(2-hydroxyphenyl-methylidene) imidazo[2,1-b][1,3,4]thiadiazole-5-carbohydrazide demonstrated marked effects on anovarian cancer cell line (Terzioglu and Gursoy, 2003), 3-[[(6-chloro-3-phenyl-4(3H)-quinazolinone-2-yl)mercaptoacetyl] hydrazono]-5-fluoro-1H-2-indolinone showed most favorable cytotoxicity against a renal cancer cell line (Gursoy and Karali 2003), 5-chloro-3-phenylindole-2-carboxylic acid(4-nitrobenzylidene)hydrazide was found to be highly active in a standard growth inhibition assay in breast cancer cells (Zhang et al., 2004) and 6-amino-4-aryl-2-oxo-1-(1-pyrid-3-yl- or 4-yl-ethylidene-amino)-1,2-dihydropyridine-3,5-dicarbo-nitrile series exhibited a high percentage of tumor growth inhibition in all cancer cell lines (Gursoy and Guzeldemirci-Ulusoy, 2007).

However, the hydrazone derivatives synthesized by scientists from our university have not been studied on their chemopreventive activity. Furthermore, previous chemopreventive studies have not focused on the head and neck region, an important site of cancer development in many parts of Asia. Therefore in this preliminary study, we have chosen one of the hydrazone derivatives, 1-[(bromomethyl)(phenyl)methyl]-2-(2,4-dinitrophenyl) hydrazine (Figure 1) to investigate its chemopreventive effect using tongue cancer cells as *in vitro* model.

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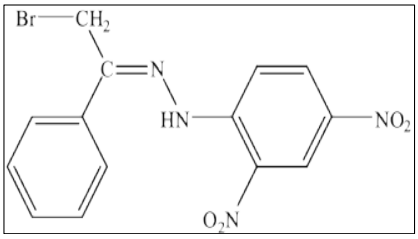

Figure 1. The Molecular Structure of 1-[(Bromomethyl) (phenyl)methyl]-2-(2,4-dinitrophenyl)hydrazine

Materials and Methods

Chemical

The hydrazone derivative (Figure 1) was prepared in stock concentration of 30 mg/ml with dimethyl sulphoxide (DMSO) and stored at 4°C. The stock solution was further diluted with the appropriate culture medium immediately before use.

Cell line and cell culture

All of the cell lines were purchased from the American Type Culture Collection in Manassas, Virginia, United States and grown at 37°C in a 5% CO_2 atmosphere. Cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM). Human tongue squamous cell carcinoma (SCC-15) cells were maintained in DMEM/F12 and normal mouse skin fibroblast (L929) cells were maintained in DMEM-HG. All media were supplemented with 10% fetal bovine serum (FBS), 100μg/ml penicillin and 100μg/ml streptomycin.

Determination of inhibitory concentration (IC_{50})

Tongue cancer cells were seeded at a density of $1x10^5$ cells per well in 6-well plates. After 24 hours of incubation in the appropriate medium, cells were treated with various concentrations of the h ydrazone derivative for another 72 hours of culture. Number of viable cells was counted under an inverted microscope using hemocytometer by Trypan Blue Exclusion Assay. The inhibitory concentration (IC_{50}) was defined as concentration of drug causing 50% inhibition in absorbance compared with untreated control cells and was determined from the graph plotted. All of the experiments were done in triplicate.

Cell proliferation assay

Tongue cancer cells were seeded in 6-well plates at a density of $1x10^5$ cells per well. After 24 hours, the Hydrazone derivative at the inhibitory concentration (IC_{50}) was added to the wells. Untreated control cells were exposed to appropriate culture medium without the compound. Cell incubation was extended for eight days and the number of viable cells was counted under an inverted microscope using hemocytometer by Trypan Blue Exclusion Assay on Day 1, Day 3, Day 6 and Day 8. The growth pattern of the untreated control and treated cells was determined from the graph plotted. All of the experiments were done in triplicate.

Cell morphological alterations and live-death analysis

Untreated control and the hydrazone derivative treated tongue cancer cells were examined for morphological changes by inverted phase contrast microscope during the determination of inhibitory concentration (IC_{50}) and cell proliferation assay. For identification of live and dead cells, cells were stained with ethidium homodimer-1 and calcein AM and examined under confocal laser scanning microsope. Calcein is well retained within live cells, producing a green fluorescence in live cells. Ethidium enters cells with damaged membranes, thereby producing a red fluorescence in dead cells.

Flow cytometric analysis

Exponentially growing cells were treated with the hydrazone derivative at the inhibitory concentration (IC_{50}) for 72 hours. Specimens were collected and prepared in triplicate by FITC Annexin V Apoptosis Detection Kit I for apoptosis analysis and by Cycle TEST PLUS DNA Reagent Kit for cell cycle analysis and analyzed by flow cytometer (BD FACSCanto II) immediately.

Cytotoxicity test

Approximately $1x10^5$ normal mouse skin fibroblast cells were seeded in each well of 6-well plates. After incubation overnight, the Hydrazone derivative at the inhibitory concentration (IC_{50}) was added and cells incubated for 72 hours. Untreated control and treated cells were examined for morphological changes by inverted phase contrast microscope. Number of viable cells was counted under an inverted microscope using hemocytometer by Trypan Blue Exclusion Assay. The viability of the untreated control and treated cells was compared and the cytotoxicity of the compound on the cells was determined.

Statistical analysis

Data values were expressed as means ± SD. Differences were compared by one-way analysis of variance (ANOVA) followed by Bonferroni correction. *P values* were considered to be statistically significant when $P < 0.05$.

Results

Determination of inhibitory concentration (IC_{50})

The hydrazone derivative showed a dose-dependent inhibitory effect on the growth of tongue cancer cells with inhibitory concentration (IC_{50}) value of 0.01 mg/ml. The result of inhibitory activity of the compound against tongue cancer cells was shown in Figure 2.

Cell proliferation assay

Treatment with the hydrazone derivative at inhibitory concentration (IC_{50}) suppressed significantly the viability of the cells in a time-dependent manner until eight days. As shown in Figure 3, the compound was an anti-proliferative agent on the tested cell line.

Cell morphological alterations & live-death analysis

Exposure of the tongue cancer cells to the hydrazone derivative resulted in alterations of cell morphology including vacuolization and cellular shrinkage (Figure 4). On live-death analysis under confocal microscopy

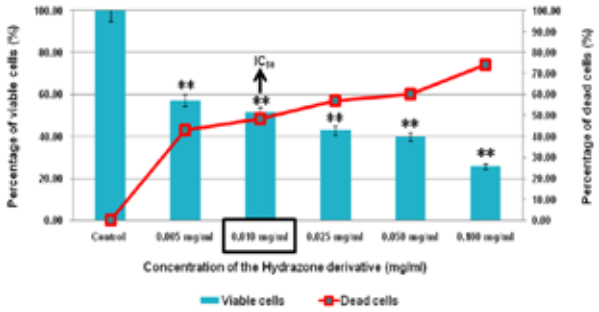

Figure 2. Concentration Dependence of Hydrazone Effects on Cell Viability**vs Control: $p<0.01$, ANOVA, Bonferroni

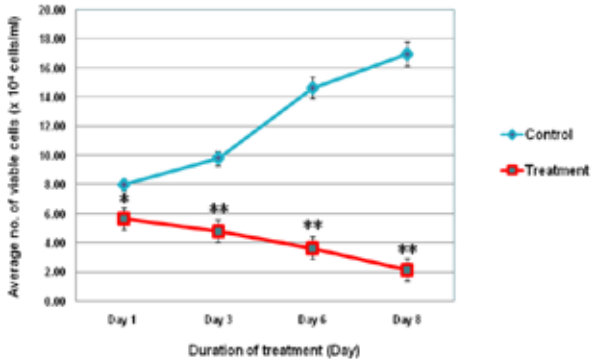

Figure 3. Anti-proliferative Effect of the Hydrazone Derivative *,** $p<0.05$, $p<0.01$, ANOVA, Bonferroni

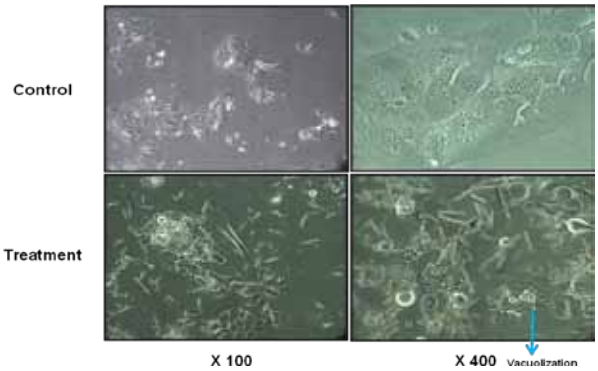

Figure 4. Effects of the Hydrazone Derivative on the Tongue Cancer Cell Morphology

using calcein and ethidium staining, it was confirmed that the compound exerted cell death (Figure 5). Presence of numerous dead cells which were stained red was observed in the sample treated with the compound.

Flow cytometric analysis

The apoptosis-inducing activities of the Hydrazone derivative in tongue cancer cells were characterized by the flow cytometric analysis assay shown in Table 1. The compound was identified as an inducer of apoptosis in the cells, as shown in Figure 6 by a two-fold increase of apoptotic activity in the early and late apoptosis stages in cells treated with the compound. The cell cycle patterns were also analyzed by flow cytometry. An increase in the G0G1 phase and decrease in the G2M phase DNA content in cells treated with the compound were observed as shown in Figure 7. The results showed that treatment of cells by the compound resulted in DNA arrest in the G0G1 phase.

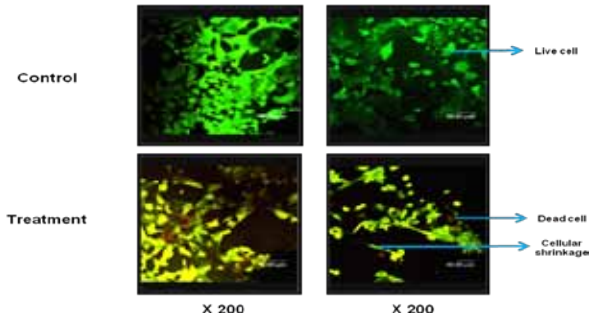

Figure 5. Identification of Live and Dead Cells by Confocal Microscopy

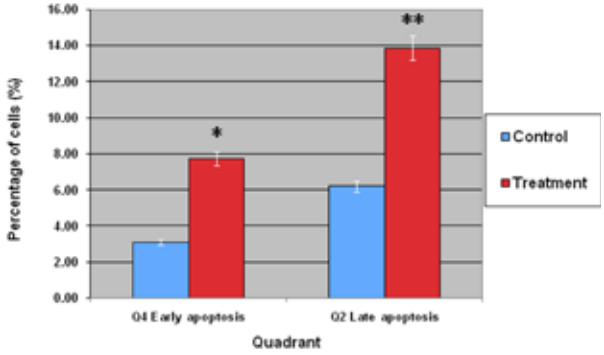

Figure 6. Increase of Apoptotic Activity in the Early and Late Apoptosis Stages in Treated Cells*,** $p<0.05$, $p<0.01$, ANOVA, Bonferroni

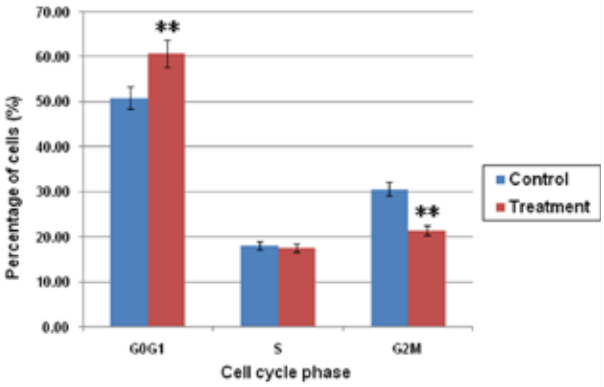

Figure 7. Increase in G0G1 Phase and Decrease in G2M Phase DNA Content of Treated Cells **$p<0.01$, ANOVA, Bonferroni

Cytotoxicity test

The cytotoxic activity of the Hydrazone derivative was determined on normal mouse skin fibroblast cells. A viability of 97.5% of cells treated with the compound was observed, which is not significantly different from the viability of untreated control cells. Absence of effect of the compound on the cell morphology and cell number clearly showed that the compound is non-cytotoxic to the normal cells at an inhibitory concentration (IC_{50}).

Table 1. Distribution of Cells in Various Stages in Apoptosis Analysis

	Percentage of cells (%)			
	Q1	Q3	Q4	Q2
	Debris	Viable	Early apoptosis	Late apoptosis
Control	1.20	89.57	3.10	6.20
Treatment	1.67	76.73	7.73*	13.87**

*,** $p < 0.05$, $p < 0.01$, ANOVA, Bonferroni

Discussion

Oral cancer is the eleventh most common cancer worldwide and in south-central Asia, it ranks among the three most common types of cancer. Over 90 percent of all oral malignancies are squamous cell carcinoma, which arise from the oral mucosal lining. Oral cancer causes considerable morbidity and is associated with a five-year survival rate of less than 50 percent. Furthermore, the survival rate for oral cancer has remained essentially unchanged over the past three decades. These alarming statistics on oral cancer have led to our interest in investigating the effectiveness of this newly synthesized Hydrazone derivative in oral cancer chemoprevention by using tongue cancer cells as *in vitro* model.

The Hydrazone derivative tested has been identified as a potent anti-proliferative agent and apoptosis inducer on tongue cancer cells. Besides, it was also found to arrest the cells in the G0G1 phase of cell cycle as measured by flow cytometric analysis assay.

The compound exhibited strong anti-proliferative activity on the tongue cancer cells with inhibitory concentration (IC_{50}) value of 0.01 mg/ml (0.03 μM). The value was compared to the inhibitory concentration (IC50) values of other Hydrazone derivatives tested on different cell lines. Several benzo[d]isothiazole hydrazones have been tested for anti-tumoral activity and were found to exhibit inhibitory concentration (IC_{50}) values against various cell lines ranging between 0.5 and 8.0 μM (Vicini et al., 2006). Hydrazinopyrimidine derivatives demonstrated inhibitory effects on the growth of a wide range of cancer cell lines generally at 0.1 and 10.0 μM median growth inhibition concentrations (Cocco et al., 2005). The compound tested demonstrated a lower inhibitory concentration (IC_{50}) compared to the other Hydrazone derivatives studied, thus acting as a potent anti-proliferative agent on tongue cancer cells. Besides, it showed stability of effectiveness in proliferation inhibition of the cells until eight days of in vitro study. The difference between the number of viable cells in treated and untreated samples was as high as 87 percent, with cell viability of only 13 percent in treated samples on the eight day.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell morphological changes and death. These changes include blebbing, vacuolization, loss of cell membrane asymmetry and attachment, cellular shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Therefore, induction of vacuolization and cellular shrinkage to the treated cells as observed in our study are significant features of apoptotic cell death.

Defects of apoptosis pathways and the ability to evade cell death is one of the hallmarks of cancers, which results in uncontrollable tumor cell growth, as well as tumor resistance to chemotherapeutic treatment (Thompson, 1995). Therefore, the discovery and development of apoptosis inducers as new chemopreventive agents is a promising approach and has been a focus of research recently. The compound tested was found to induce a two-fold increase especially in early apoptotic activity of tongue cancer cells. Previous studies have addressed the role of other Hydrazone derivatives on apoptosis induction, where 5-methyl-3-phenylindole-2-carboxylic acid(4-methylbenzylidene)hydrazide and 5-chloro-3-phenylindole-2-carboxylic acid(4-nitrobenzylidene) hydrazide showed a 20-fold increase of apoptotic activity in breast cancer cells (T47D) (Zhang et al., 2004). The different level of apoptosis induction could be attributed to the difference in Hydrazone derivative used, dosage of compound, duration of treatment and cell line tested.

In similar study, 5-chloro-3-methylindole-2-carboxylic acid(4-nitrobenzylidene)hydrazide showed arrest of breast cancer cells (T47D) in G2M phase of the cell cycle. The compound and its analogs were found to inhibit tubulin polymerization, suggesting that inhibition of microtubule assembly most probably is the primary mechanism of action of these compounds (Zhang et al., 2004). On the other hand, our results have shown that the compound tested delayed cell transition from the G0G1 phase and prolonged the duration of the G0G1 phase, thus inducing a G0G1 phase cell cycle arrest and G2M phase reduction. However, further study has to be done to determine the most probable primary mechanism of action of the compound in inducing the G0G1 phase cell cycle arrest. *In vitro* tubulin polymerization assay could be conducted to determine if the compound has any effect on tubulin polymerization as proven in other experiment. Nonetheless, the interference with microtubule function represents an important mechanistic principle in anti-cancer drug discovery that underlies the anti-tumor activity of a variety of clinical validated anti-cancer agents (Jordan, 2002) such as taxanes, a class of microtubule stabilizer and vincas, a class of microtubule depolymerizer.

In conclusion, the Hydrazone derivative is a potent anti-proliferative agent in the tongue cancer cells. The findings suggested that the compound exhibited anti-cancer activity via apoptosis induction and cell cycle regulation pathway. This research has led to real progress in oral cancer chemoprevention, as previous chemopreventive studies have not focused on the head and neck region. Nonetheless, much work still needs to be done to assess the signaling pathway involved, systemic anti-proliferative effect and potential therapeutic promise of the compound *in vivo*. Continuing research of the potentiality of the Hydrazone derivative as chemopreventive agent offers hope that, in the future, even more people with this disease will be treated successfully and people with oral cancer can look forward to a better quality of life.

Acknowledgments

The authors acknowledge the fine technical support given by authority and staff of Craniofacial Laboratory, School of Dental Sciences and Department of Immunology, School of Medical Sciences, Universiti Sains Malaysia.

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