The Potential of 9,10-Anthraquinone in Inhibiting Human Cancer Cells Growth

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ABSTRACT

Background: 9,10-Anthraquinone (9,10-AQ) is a contaminant on some agricultural products and considered as carcinogenic based on EU Regulation No. 1146/2014. Except for little evidence on experimental rats, there is no strong proof regarding the carcinogenicity in humans. Therefore, it is essential to find a safe dose of this compound since the difference in 9,10-AQ levels will affect cancer cell growth. This research aims to find the 9,10-AQ concentration that does not proliferate the human cancer cells under in vitro study.

Methods: In determining the 9,10-AQ concentration that does not proliferate the cancer cells growth, 0.01 to 500 mg/L 9,10-AQ was directly tested on four human cancer cells (colorectal carcinoma HCT 116, colon adenocarcinoma WiDr, breast cancer MCF-7, and cervical cancer HeLa), and the viability of the cells was counted via (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. In the gene expression level, the effects on a selected cancer cell line were determined by qRT-PCR against *BAX*, *BCL-2*, *PCNA*, and *P53*.

Results: The result indicates that 9,10-AQ up to 500 mg/L concentration does not proliferate the cell's growth but instead inhibits those four cancer cells' growths. The concentration of 9,10-AQ that inhibits 50% the cancer cells growth (IC₅₀) value was 321.8 mg/L (1.55 mM) against HCT 116 and above 500 mg/L (above 2.40 mM) against WiDr, MCF-7, and HeLa. The 9,10-AQ at 500 mg/L (or 2.40 mM) increases *BAX* expression and acts as an apoptotic agent on HeLa cells.

Conclusions: The investigation has shown that 9,10-AQ up to 500 mg/L concentration does not proliferate the cancer cell growth; instead, it inhibits the HCT 116 and HeLa cells growth. We have preliminary evidence regarding the apoptotic mechanism of 9,10-AQ by increasing *BAX* gene expression on HeLa cells.

INTRODUCTION

The 9,10-anthraquinone (9,10-AQ) is a compound formulated with insecticides and fungicides and acts as bird repellent [1]. Its presence in agricultural products, such as in fruit, vegetable, oil, spices, and animal product in European Union, is prohibited according to European Food Safety Authority (EFSA) [2]. Therefore, this synthetic compound is classified as Carc 1B based on some sufficient evidence for animal carcinogenicity [3,4].

The maximum residue level (MRLs) of 9,10-AQ is currently set to be 0.01 mg/kg, which applies to agricultural products such as fruit including pine fruit, citrus fruit, stone fruit, root and tuber, vegetable

including bulb vegetable, fruiting vegetable, brassica vegetable, leaf vegetable, legume, stem vegetable, fungi, seaweed, cereal, sugar plant, and animal products. The MRL is slightly higher for tree nuts, oilseeds, oil fruit, tea, coffee, herbal infusion, cocoa, spices seed, rhizome, and bud, which is 0.02 mg/kg. Nonetheless, the EFSA admits that due to the lack of toxicological data on 9,10-AQ, it is impossible to assess whether a standard 0.01 mg/kg MRL, as defined by Regulation (EC) No 396/2005, would provide adequate consumer protection in the event of abuse [2]. The carcinogenicity of 9,10-AQ in humans has not been widely reported. The only reports on chronic neurotoxic symptoms due to 9,10-AQ in humans are visual impairment, acute oral poisoning, and skin irritation [5,6].

Cancer cell lines are typical for in vitro model systems to assay a compound's effect on cancer cell growth, such as determining whether the compound is proliferating the cell (has carcinogenic potency) or inhibit cell growth (has antiproliferation potency). One advantage of using cell culture is its consistency that can be obtained. It is known that the concentration difference applied to the cells will have a different effect. Thus, it is essential to determine the 9,10-AQ concentration that does not proliferate the cancer cell growth. This paper presents, for the first time, some experimental results of directly administering several doses of 9,10-AQ to the development of four types of human cancer cells, namely (1) human intestinal cancer cells HCT 116 (ATCC CCL 247), (2) WiDr human intestinal cancer cells (ATCC CCL 218), (3) MCF-7 human breast cancer cells (ATCC HTB 22), and (4) HeLa cervical cancer cells in humans (ATCC CCL 2). Through this experiment, the 9,10-AQ dose that caused cancer cells' proliferation, and the safe dose (same level as the control) will be disclosed. The mechanism of the 9,10-AQ through *BAX*, *BCL2*, *P53*, and *PCNA* expression on selected cancer cell lines will be discussed..

METHODS

Evaluation of cytotoxicity based on tetrazolium dye (MTT) assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of the 9,10-AQ. The protocol relied on the yellow tetrazolium salt's cleavage, MTT, forming a soluble blue formazan product by mitochondrial enzymes. The quantity of formazan was proportional to the population of living cells present under MTT exposure [7]. The tested cancer cells were human colorectal carcinoma HCT 116 (ATCC CCL-247), human colon adenocarcinoma WiDr (ATCC CCL-218), human breast cancer MCF-7 (ATCC HTB-22), and human cervical cancer HeLa (ATCC CCL-2).

The cells were cultured in a medium containing Dulbecco's modified eagle medium (DMEM, Gibco USA), 10% fetal bovine serum (FBS, Gibco USA), and 1% antibiotic (Penicillin Streptomycin, Invitrogen USA) in a favorable environment (37 °C, 5% CO₂, 90% humidity). The cells were sub-cultured and seeded into a 96-well tissue culture plate with 5000 cells/well to adhere for 18-20 hours in a growth medium; each concentration was performed in triplicate. The following day, the medium was removed from the wells and replaced with 9,10-AQ (TCI India, CAS No. 84-65-1) in a growth medium. Dimethyl sulfoxide (DMSO) was added to facilitate the sparingly soluble 9,10-AQ's homogeneous solution, with the final concentration of DMSO 0.5% in

each well. The plates were then incubated with 9,10-AQ in various concentrations: 500, 250, 150, 25, 2, 0.02, and 0.01 mg/L for 48 hours. Tetrazolium salt, MTT (Sigma USA), was added to each well in the plates (10 µL of 5 mg/mL in PBS). The plates were further incubated for 4 hours. The medium was then removed, and 1 N HCl in isopropanol (100 µL) (Merck, USA) was added. Finally, the absorbance at λ 562 nm of the plates were read using a microplate reader (BioRad USA). The absorbance values were blanked against 1 N HCl in isopropanol, and absorbance of the cells exposed to the respective medium was taken as 100% cell viability (taken as negative control). The 50% inhibition concentration (IC₅₀) of 9,10-AQ against each cell was determined based on the correlation between percent inhibitions and the concentrations. The concentration on the correlation that gives 50% inhibition is the IC₅₀ value.

The HeLa cells were treated with 9,10-AQ at 250 mg/L and 500 mg/L; in this study, untreated cells were used as a control. The concentrations of 9,10-AQ used in this study for cell exposure were chosen from a previous MTT assay result. Treated cells and untreated cells were incubated for 48 hours at 37°C in 95% humidity and 5% CO₂ (Forma Series II Water-Jacketed CO₂ Incubator, Thermo Fisher Scientific, Waltham, MA). According to the manufacturer, mRNA was extracted from cells using the RNeasy kit (Qiagen, Germany) and reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen, USA) accordingly. We employed the quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) in measuring the expression of markers for apoptosis marker (*BAX*, *BCL2*, *P53*), proliferation markers (*PCNA*); *GAPDH* was used as the internal calibrator gene. **Table 1** displays the sequence of primers used. Reactions were performed using SsoFast™ EvaGreen® Supermix (Biorad, Hercules, Un) on the iCycler iQ5 (Biorad, USA). Relative gene expression followed the delta-delta threshold method (2^{-ΔΔCT}) with the CT gene target normalized with CT reference (*GAPDH* gene) [8].

Statistical Analysis

Data are presented as average from triplicate data for % inhibition and gene expression. The % inhibition and gene expression of treatment were compared with control cells using t-tests at α = .05.

RESULT

The 9,10-AQ's effect is diverse in the growth of the cancer cell lines (**Figure 1**). Surprisingly, a high concentration of 9,10-AQ (25–500 mg/L) inhibits the cell growth for HCT 116 and HeLa cells, but cell growth inhibition for WiDr and MCF-7 cells are found at sufficiently high concentration (250 and 500 mg/L).

Table 1. Primers sequences

Marker	Sequence (5' – 3')		References
	Forward	Reverse	
<i>BAX</i>	CCCGAGAGGTCTTTTCCGAG	CCAGCCCATGATGGTTCTGAT	[9]
<i>BCL2</i>	GCTCTAAATCCATCCAG	CCTCTCCATCATCAACTT	[9]
<i>P53</i>	GAGCTGAATGAGGCCTTGA	CTGAGTCAGGCC CTCTGTCT T	[10]
<i>PCNA</i>	GAAGCACCAAACAGGAGAA	TATCGGCATATACGTGCAAA	[11]
<i>GAPDH</i>	CGGATTGGTCTGATTGG	TCAAAGGTGGAGGAGTGG	[12]

Figure 1. The average inhibition (%) of cancer cell line growth of HCT 116, WiDr, MCF-7, and HeLa by the 9,10-AQ at the concentrations of 0.01 to 500 mg/L. *: significantly different from the control cell ($P < 0.05$)

Lower concentrations (< 25 mg/L) inhibit less than 30% of the cancer cell line growth. The percentage inhibition displays an increasing trend on HCT 116 cells, and HeLa cells align with the increasing concentrations. The 9,10-AQ does not seem to affect MCF-7 cells and the WiDr cells; it inhibits below 20% in all concentrations used in this study. The inhibition of 9,10-AQ on HeLa cells growth is about 50% at a concentration of 250 and 500 mg/L; therefore, the HeLa cells were selected to be further used as a molecular assay model.

Based on cell proliferation inhibition upon 9,10-AQ concentrations, the concentration that can inhibit 50% cell growth (IC_{50} value) is determined (**Table 2**). The corresponding IC_{50} values on WiDr, MCF-7, and HeLa cells are above 500 mg/L, and the IC_{50} value on HCT 116 is approximately 320 mg/L.

Table 2. Inhibition concentration of 50% cancer cell growth (IC_{50}) of anthraquinone

Cancer cells	IC_{50} (mg m L-1)	IC_{50} (mM)
HCT 116	321.8	1.55
WiDr	>500	>2.40
MCF-7	>500	>2.40
HeLa	>500	>2.40

This study also provides additional physical information. Specifically, upon treatment with 9,10-AQ, cell mortality starts at 500 mg/L and confirms abnormality in cell morphology (**Figure 2**). They showed differences in morphology (demonstrated with the arrow on each figure) resulting in the loss of cell–cell contact (A1), plasma membrane destruction (A2), cell disruption (C1), the formation of apoptotic bodies (D1), and cell shrinkage (D2), depending on treatment concentration. The cell control has typical morphological specifications until the completion of the incubation of treatment. On the contrary, with a 500 mg/L concentration of 9,10-AQ, at 0.01 mg/L, the cell growth is not significantly different from the control cells, meaning that the cells still grow well but do not show any proliferation.

To explore the 9,10-AQ's possible mechanisms as an antitumor, we experimented on the level expression of *BAX*, *BCL2*, *PCNA*, and *P53* on HeLa cells at a 9,10-AQ concentration of 250 and 500 mg/L. The result showed that the *BAX* and *BCL2* level expression increases in the presence of 9,10-AQ 250 and 500 mg/L, but the increase of *BAX* expression is higher than the increase of *BCL2* level expression (**Figure 3**). The *BAX* level expression increases about 3.6 and 5 times higher than the control for 9,10-AQ at a 250 and 500 mg/L concentration. Contrary to the increase of the *BAX* level expression, *BCL2* level expression only increased by

approximately 40 and 20% (compared to control, respectively) for 9,10-AQ at a concentration of 250 and 500 mg/L. The level expression of *P53* is increased by about 30% compared to the control at a concentration of 9,10-AQ of 500 mg/L. In comparison, the expression of *PCNA* increases by only about 60% and 40% compared with control at 9,10-AQ concentration of 250 and 500 mg/L, respectively.

DISCUSSION

The 9,10-AQ had been classified as Carc 1B that was sufficient evidence for animal carcinogenicity [3]. The research reported that 9,10-AQ induced tumors in rats, especially in the liver, kidney, urinary bladder, and that 9,10-AQ was carcinogenic only to the liver [13]. In another report, noticeable effects were reported when the rats were administered with 9,10-AQ about 7,500 mg/L, equivalent to 555 mg 9,10-AQ/kg body weight [14]. This concentration is indeed an irrational dose (i.e., 555 mg 9,10-AQ/kg body weight of rats is equal to 89.95 mg 9,10-AQ/kg human body weight), as reported by Shin et al. [15]. It is widely known that vitamins and minerals that are good for health can be toxic if consumed at an excessive or irrational dose [16].

Figure 2. Various concentrations of 9,10-AQ induced morphological effects. A = HCT 116, B = WiDr, C = MCF-7, D = HeLa, 1 = 500 mg/L, 2 = 0.01 mg/L, and 3 = control cells

Figure 3. The level expression of *BAX*, *BCL2*, *P53*, and *PCNA* on HeLa cells induced by 9,10-AQ at 250 and 500 mg/L concentrations.

*: significantly different from the control cell ($P < 0.05$)

Our experiment confirms that 9,10-AQ at all tested concentrations do not proliferate the targeted cancer cells, i.e., human intestinal cancer cells, human breast cancer cells, and cervical cancer cells. On the contrary, cancer cell growth was adversely inhibited (**Figure 1**). The inhibition is increasing as the 9,10-AQ concentration increases. The cell morphology indicates cell mortality when the highest 9,10-AQ concentration (500 mg/L) was applied (**Figure 2**). This study follows the hypothesis that different concentrations of 9,10-AQ will affect cancer cell growth. Surprising results show that 9,10-AQ is a potent proliferation inhibition agent, especially against HCT 116 with the IC₅₀ of 1.55 mM. Our findings also support minor disturbance on chronic neurotoxic symptoms due to 9,10-AQ in humans [5,6]. It is also reported that 9,10-AQ can be a potent aggregation inhibitor for Alzheimer's disease [17]. Rhein, an AQ natural product, is also reported as a potential antitumor toward the HeLa cancer cell with IC₅₀ of 1.3–40.0 μ M [9]. Other AQ derivatives are also reported as potential anticancer against several cancer lines [8].

Since the 9,10-AQ demonstrates antiproliferation activity against the HeLa cancer cell, the mechanism of action as antiproliferation was further investigated at a molecular level by determining the *BCL-2*, *BAX*, *PCNA*, and *P53* gene expression. *BCL-2* (B-cell lymphoma 2) is a transmembrane protein that regulates apoptosis (cell death). *BCL-2* promotes cellular survival and inhibits proapoptotic proteins, including *BAX*, *BAK*, *BAD*, and

BIM [10-12,18]. In the presence of *BCL-2* as an antiapoptotic, *BAX*'s activation will decrease, and the death signal disappears. This study exhibits that 9,10-AQ significantly up-regulates *BAX* expression (by approximately 3–5 folds) but does not change the expression of *BCL-2*, which implies a sudden rise of *BAX/BCL-2* ratio in 9,10-AQ treated HeLa cervical cancer cell line about 2.6 and 4.3 folds compared with control cells. It means 9,10-AQ plays a role in the apoptosis of HeLa cell lines.

Proliferating cell nuclear antigen (*PCNA*) is an acidic nuclear protein related to DNA replication on the transition from the G1 phase to the S phase and connected with tumor cells' proliferation [19]. *PCNA* is an indicator to evaluate the state of cell proliferation [20]. When the expression of *PCNA* increases, it means cell proliferation increases as well. Our findings show that the increase in *PCNA* expression is not significant compared to those of control, which means that the 9,10-AQ does not substantially proliferate HeLa cancer cells.

The *P53* gene is a tumor suppressor that increases during DNA impairment [21,22]. This gene also contributes to the G1 phase regulation of the cell cycle. Our results show that the expression of *P53* does not increase significantly, only about 30%, even after the administration of 500 mg/L of 9,10-AQ (**Figure 3**). Thus, there is no confirming evidence that below the safe dose, the 9,10-AQ does not harm the DNA.

CONCLUSIONS

The effect of 9,10-AQ's direct interaction on four different cancer cells, namely human colorectal carcinoma HCT 116, human colon adenocarcinoma WiDr, human breast cancer MCF-7, and human cervical cancer HeLa, demonstrates to inhibit the four cancer cells growth. The 9,10-AQ acted as an inhibition agent against the HeLa cancer cell line by increasing the *BAX* ratio to *BCL-2* expression via the apoptosis mechanism. We, therefore, suggest that 0.01 and 0.02 mg/kg of 9,10-AQ as MRL in agricultural product commodities, including tea, be reconsidered.

DECLARATIONS

Competing of Interest

The author(s) declare no competing interest in this study.

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