Thesis Body

Chapter 1: Introduction

1.1. Problem background & formulation

Nasopharyngeal carcinoma (NPC) is one of the neck and head cancer which, despite being a rare human malignancy worldwide, it is relatively prevalent in geographical regions such as North Africa, Southeast Asia, and China. Its incidence can reach up to 40 in 100,000 individuals (Wang et al., 2016). There were approximately 129,000 of new NPC cases worldwide in 2018 by which its mortality rate reached up to 56% (Bray et al., 2018).

Most NPC diagnoses are attained at advanced stages, i.e. more than 70% of patients diagnosed with NPC were already at an advanced stage (Tang et al., 2016). The clinically silent and unspecific symptoms-bearing NPC has been identified as a major underlying cause for diagnosis at an advanced stage (Wang et al., 2017). Meanwhile, significant differences in overall survival are observed in the early stages compared to advanced stages of NPC. Up to 98% of stage I NPC patients could reach 10-year survival, 60% of stage II NPC patients could reach 10-year survival, whereas the median survival for advanced-stage NPC patients dropped down to 3 years (Wu, Li & Pan, 2018).

The standard conventional therapies for solid tumors, including NPC nowadays involve surgical excision of the tumor following chemotherapy and adjuvant radiation therapy, which causes various adverse side effects, significantly crippling quality of life. Radiotherapy (RT) has been one of the standard treatments for non-metastatic NPC, particularly having high cure rate for early stages of NPC (Paiar et al., 2012; Zhang et al., 2013). In the case of late stages of NPC though, often various modes of combined chemoradiotherapy (CRT) would be needed to combat the complications (Paiar et al., 2012; Zhang et al., 2013). Nevertheless, outcomes for locoregionally advanced NPC is still immensely undesirable, generally bearing high possibility for local treatment failure and distant metastases (Wei & Kwong, 2010; Paiar et al., 2012; Zhang et al., 2013). Moreover, occurrences of subsequent toxicity/side effects are still well documented in these therapies, and even more adverse

in cases of recurrence and distant metastasis (Liu et al., 2019). In addition to the detrimental side effects, accessibility and cost of such treatment mode would only restrict patients with a far-from-ideal option (Arora et al., 2010; Shin et al., 2018). Hence, an alternative approach for cancer therapies would be very beneficial towards modern human healthcare.

Currently, the main modes of cancer therapies include chemotherapy, radiotherapy, and surgery. Alternative anticancer agents from natural compounds have as well gained a fair amount of interests for some of its better anticancer efficacy and lesser side effects compared to the conventional cancer therapies. The preclinical studies would be an essential initial step in the development of any anticancer agents such that its assessment of its chemical properties, anticancer effects, as well as the toxicity it possesses would be crucial for subsequent anticancer agent development protocols. The preclinical studies typically go through multiple obligatory steps: 1) selection and acquisition of compounds, 2) screening for antitumor activity, 3) anticancer production and formulation, and 4) animal toxicology (Schwartsmann, Winograd & Pinedo, 1988). Various preclinical methods in determining anticancer effects of a candidate anticancer agent include the utilization of human cancer cell lines *in vitro,* tumor xenograft models *in vivo,* and genetically engineered mouse models (Kumar, Bajaj & Bodla, 2016). The oncogenic phenotypic change (proliferation, migration, invasion, etc.) of cancer cells is one of the most utilized parameters to be tested in preclinical anticancer studies as it reflects the overall major components of cancer cells' behavior and processes. Availability of well-characterized cancer cell lines enables screening assays on testing cancer cell phenotypes of candidate anticancer agents (HogenEsch & Nikitin, 2012).

The use of phytochemicals/use of medicinal plants has been viewed as an increasingly prominent candidate of cancer therapeutics, particularly the realistic potential to be an ideal chemopreventive agent. Phytochemicals have been used as traditional treatments for countless human diseases for centuries. As in 2011 itself, it is estimated that 70-95% of the populations in most developing countries still rely on traditional medicines for primary care, whereas industrialized

nations are still reported that 70-90% of the populations have used traditional medicines as complementary or alternative modalities (Robinson & Zhang, 2011). *Figure 1.1* shows the characteristics which should be found on an ideal chemopreventive agent. Despite that it would be highly unlikely for a single chemopreventive agent to possess all these characteristics, such as phytochemicals does have prominent properties including plant metabolites with wide range of biological functions with null or considerably lesser side effects, compared to some of the conventional standard cancer therapies such as chemotherapy and radiotherapy (Arora et al., 2010; Shin et al., 2018). It is even more desirable in scenarios where no cure is available in certain cancers, or due to the costly and limited access to modern cancer therapies.

Asiatic acid (AA) is one of the extensively studied phytochemicals which can be found in the herbaceous plant, *Centella asiatica.* Some of the earliest studies have proven its therapeutic effects such as neuroprotective, cardioprotective, antihypertensive, wound healing, ulcer-protective, antimicrobial, antiviral and immunomodulatory potential (Brinkhaus et al., 2000; Meeran et al., 2018). Countless studies followed after in demonstrating promising anticancer properties of AA in various cancers characterizing each target components of the cancer cell mechanisms (Meeran et al., 2018). Moreover, asides from its biological effects and improving the quality of life, it is also well known for its excellent bioavailability and the little to no side effects it induces (Mato et al., 2011). In recent years, studies have exhibited significant improvement in therapeutic effects of AA through chemical modification of the AA backbone, also known as the AA derivative (Lv et al. 2018). Hence, there has since been an appreciable interest towards studies in the synthesis and evaluation of AA derivatives for the development of a novel and effective anticancer treatments. Moreover, there have not yet been studies of anticancer effects in AA nor AA derivatives in NPC, this study provides great interest to initiate and pioneer a promising anticancer drug development in NPC.

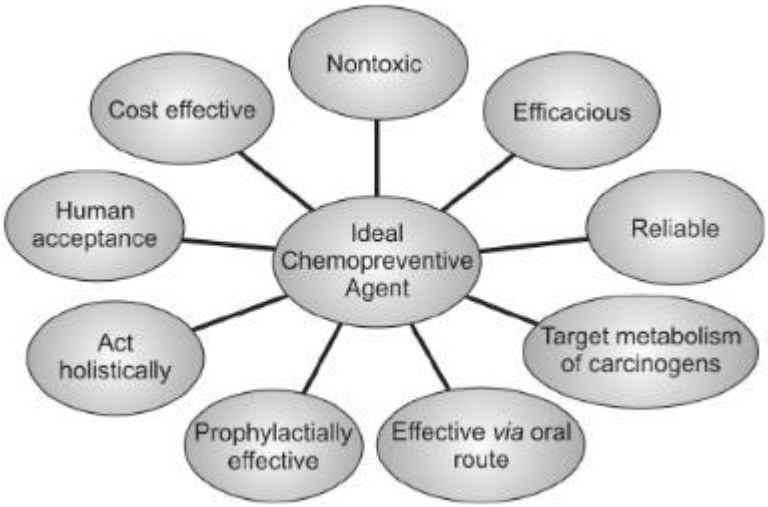

Figure 2.1) Characteristics of ideal chemopreventive agents. Adapted from: Arora et al., 2010.

1.2. Research objectives

The objectives of this research are 1) to observe the cancer-inhibiting effects of Asiatic acid (AA) and 3 of its derivatives: Asiatic acid derivative A (AA-A), Asiatic acid derivative B (AA-B) and Asiatic acid derivative C (AA-C) in NPC *in vitro* using the CNE2 NPC cell line through inhibition of oncogenic phenotypes such as cellular viability, proliferation, migration and invasion, as well as determining the most effective treatment among the 4 treatments used and 2) to elucidate any underlying mechanisms of potential cancer-inhibiting effects induced by AA and its derivatives.

1.3. Research Scope

The scope of the research of determining the cancer-inhibiting effects of Asiatic acid and 3 of its derivatives encompasses through a set of phenotypic parameters associated with oncogenic capacities such as the cellular viability, proliferation, migration, and invasion tested *in vitro* using the CNE2 NPC cell line as well as elucidating its potential underlying mechanisms. The evaluation of cellular viability, proliferation, migration and invasion were achieved collectively through MTT assay, clonogenic assay, wound healing assay, and transwell assay, whereas protein quantification to understand the involvement of the protein behind the cancer-inhibiting effects through western blot.

a. Cell Viability

MTT assay – The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is an *in vitro* bioassay technique to measure cell viability through the quantification of cellular metabolic activity in form of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce MTT into a purple-colored insoluble formazan (Kuete, Karaosmanoğlu & Sivas, 2017). The resultant insoluble formazan would be quantified in optical density (OD) using microplate reader at a wavelength of 540 nm. Hence, this colorimetric quantification of insoluble formazan presumably associates proportionally to the number of viable cells in a given well. The use of MTT assay have been relatively common in studies to determine cytotoxicity or sensitivity of certain drugs or drug screening on cell lines (Van Meerloo, Kaspers & Cloos, 2011). Mainly, in this project, the assay would provide the data of the cell viability inhibition and drug sensitivity of CNE2 cell line towards each of the treatments in order to determine the concentration of each of the treatments required to achieve 50% growth inhibition compared to the untreated control; also known as the 50% inhibitory concentration (IC_{50}). The IC_{50} concentration of each of the treatments would then set as the base of the treatment concentrations for the following experimental methodologies.

b. Colony-forming units capacity/cell proliferation

Clonogenic assay – The clonogenic assay or also known as the colony forming unit assay was performed to observe and assess the colony forming capacity/producing progeny of CNE2 cells (indicated through a single cell forming a colony of at least 50 cells) among the negative control untreated cells and the treatment control cells. This method has been deemed as successful and widely utilized in radiation biology in assessing radiation sensitivity and efficacy in particular cell lines, which could also serve in determining the effects of drug treatments and cytotoxic agents in the cells' colony forming ability (Rafehi et al., 2011). This method typically employs cell seeding of a very low cell density, treatments and conditions to each control respectively, followed by incubation usually

from 1.5 – 3 weeks according to each of the cell lines used before performing cell fixation and staining to enumerate cell colonies.

c. Cell migration

Wound healing assay – The wound healing assay is a methodology applied to study the overall collective characteristics of cellular proliferation and migration mimicking the wound healing process in a two-dimensional locomotion, also known as the sheet migration (Jonkman et al., 2014). The main idea of this methodology is simply the inducing a wound formation through mechanical force to a confluent monolayer of cells, whereby the "closing" of the wound or the wound healing formation through directional collective migration would be monitored and quantitated. The directional collective migration would take part through polarization of the microtubule-organizing centers (MTOC) and the development of lamellipodia towards the wound gap as well as the inflammatory mediators produced as a result of the wound formation (Cory, 2011; Molinie & Gautreau, 2018).

In this project particularly, incubation following the wound formation would be limited to a specific duration to which would only reflect the directional collective migration of CNE2 cells, forestalling the proliferation activity contributing to the overall wound healing process.

d. Cell invasion

Transwell assay – The transwell assay, also known as the Boyden Chamber assay, fundamentally allows the observation for the migratory actions of cells towards chemoattractant signals. This assay consisted of two-chamber systems with a microporous membrane separating each other, with each of the chambers possessing usually possessing unique microenvironment to induce the tested cell motility mechanisms such as chemotaxis, haptotaxis, and chemokinesis (Chen, 2005). Despite both wound healing and the transwell assays are useful for the analysis of cell migration, the transwell assay, specifically characterizes single-cell migration instead of collective sheet migration. This assay also provides the system to analyze the invasive capacity of cells as well; this can be

performed further through the addition of extracellular matrix (ECM) (commonly used ECM layers include matrigel and collagen) layer just above the microporous membrane layer to imitate the oncogenic process of ECM invasion and extravasation (Justus et al., 2014).

e. Western Blot

Western blot was done to quantify certain protein markers in order to determine the underlying proteins involved/targeted in the anticancer mechanism of AA and its derivatives, Claudin-1, p-Akt, and t-Akt were the protein markers chosen to be quantified due to being several of the protein prominently involved in the epithelial-mesenchymal transition (EMT) process and NPC tumorigenesis.