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Anticancer Mechanism of a Novel Quaternized Amino Glucosamine Derivative and Its Inhibitory Effects on Matrix Metalloproteinases and NF-κB

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신규 합성된 Quaternized Amino Glucosamine 유도체의 항암기전 및 기질금속단백질분해효소와 NF-κB 의 억제효능에 대한 연구

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Abstract

In the present research, cell-specific cytotoxic effect of newly synthesized quaternized amino glucosamine (QAGlc) in melanoma cells was identified. Analysis of apoptosis employing different approaches confirmed that this molecule induces apoptosis in a concentration and time-dependent manner in B16F1, melanoma cells. Treatment of QAGlc increased the promoter activity and protein expression level of p53 proving QAGlc followed a p53-dependent apoptotic pathway. Following treatment, QAGlc induced a cascade of apoptosis-related proteins including p21, PUMA, cytochrome C, caspase-9 and caspase-3. Concurrent activation of Apaf-1 greatly contributed to apoptosis induction effect of QAGlc in melanoma cells. Cell cycle analysis of B16F1 cells in the presence of QAGlc further confirmed that QAGlc was involved in cell cycle arrest at G_1 phase. This was favored by decreased protein expressions of cell cycle-related proteins such as cyclin-D, cyclin-E, cdk-2, cdk-4, cdk-6, Bcl-2 and E2F. The potency of QAGlc to affect transcriptional regulation of MMP-2 and MMP-9 enzymes was identified in HT1080 fibrosarcoma cells and it showed a concentration-dependent effect. This inhibition of MMP-2 and MMP-9 was found to work via down-regulation of both AP-1 and NF-κB transcription factors induced by PMA. Further analysis confirmed that functions of these transcription factors were correlated to suppression of mitogen-activated protein kinases (MAPKs). Moreover, it was elucidated that

suppression of NF-κB by QAGlc occurs through the inactivation of IKK as investigated in RAW264.7 immune cell study. In addition, indirect NF-κB inactivation by QAGlc was a result of suppression of MAPKs and related inflammatory cytokines.

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List of Abbreviations

AP-1	activator protein-1
Apaf	apoptosis activating factor
ASP	aspirin
cdk	cyclin-dependant kinase
cyc-C	cytochrome C
DEX	dexamethasone
DMSO	dimethyl sulfoxide
DOX	doxycycline
E2F	elongation factor 2
ERK	extracellular signal-regulated kinase
ETP	etoposide
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
G3PDH	glyceraldehydes-3-phospahte dehydrogenase
Glc	glucosamine
IKK	IκB kinase
IL-1β	interleukin-1β
IL-6	interleukin-6
JNK	c-Jun N-terminal kinase
LPS	bacterial lipopolysaccharide
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducing kinase
p38	p38 mitogen-activated protein kinase
PGE_2	prostaglandin E_2
PI	propidium iodide

PMA	phorbol myristate acetate
PUMA	p53 up-regulated modulator of apoptosis
QAGlc	quarternized amino glucosamine
Rb	retino-blastoma protein
RT-PCR	reverse transcription-polymerase chain reaction
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

Part 1

Anticancer Mechanism of Quaternized Amino Glucosamine in B16F1 Melanoma Cells

Introduction

Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Various anticancer agents with different primary intracellular targets have been identified during past few decades. However, interest is continuously growing and great efforts have been laid to identify novel anticancer target molecules and to discover effective drug candidates.

1. Therapeutic potential of cell cycle arrest and checkpoint control in treatment of cancer

Among number of proposed methods to cure cancer, many studies have shown an association between cell cycle regulation and treatment of cancer. In recent years, inhibition of the cell cycle has come to be appreciated as a target for the management of cancer (Buolamwini *et al.*, 2000; Collins *et al.*, 1997; Hajduch *et al.*, 1999; McDonald *et al.*, 2000; Sherr *et al.*, 1996; Sherr *et al.*, 1999). Anticancer agents may alter regulation of the cell cycle machinery, resulting in an arrest of cells in different phases of the cell cycle and thereby reducing the growth and proliferation of cancerous cells. Components of the cell cycle machinery are frequently altered in human cancer cells. Central players are the cyclin-dependent kinases (cdks), which govern the initiation, progression, and completion of cell cycle events. The scheduled activity of the cdks, which allows orderly transition between cell cycle phases, is controlled by their association with cyclins and cdk inhibitors, by their state of phosphorylation, and by ubiquitin-mediated proteolysis. As malignant cells evolve, both genetic and epigenetic mechanisms commonly affect the expression of cell cycle regulatory proteins, causing overexpression of cyclins and loss of expression of cdk inhibitors. A major consequence is deregulated cdk activity, providing cells with a selective growth advantage. The crucial role of the cdks has prompted great interest in the development of specific

kinase inhibitors that would be expected to block cell cycle progression and induce growth arrest.

Another hallmark of the transformed state is incompetent checkpoint control, resulting in aberrant responses to cellular damage. For example, damage to DNA or the spindle apparatus normally triggers cell cycle arrest or apoptosis, depending on the degree of damage and the cellular context. Cell cycle arrest most frequently occurs at the G_1/S or G_2/M boundaries. When checkpoint arrest control is compromised, initiation of S phase or mitosis occurs despite cellular damage, and the ensuing genetic instability may lead to the eventual emergence of a malignant clone. However, this failure of cell cycle arrest responses in malignant cells can also be exploited therapeutically. Cells in which checkpoint control is disrupted are more sensitive to additional genotoxic or microtubular damage. Unbridled cell cycle progression in the presence of such damage is usually lethal, which may explain the selective sensitivity of some cancer cells to DNA-damaging treatments. For this reason, intact components of cell cycle arrest checkpoints are also potential targets for novel antineoplastics, and their inhibition may increase the sensitivity of tumor cells to standard chemotherapy and radiation (Hartwell *et al.*, 1994).

Many of the compounds under study as anti-tumor agents act at multiple steps in the cell cycle, and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of the target cells. Hence, an understanding of the molecular interactions involved may suggest ways to sensitize cells to the effects of these compounds. In particular, combinations of drugs, applied in a specific sequence, may be used to maneuver a tumor cell population into a state where it is most susceptible to the cytotoxic effects of novel, or indeed traditional, chemotherapeutic agents.

2. Therapeutic potential of the induction of apoptosis in treatment of cancer

Furthermore, in recent years, programmed cell death, i.e., apoptosis, which is a phenomenon associated with many physiological and pathological processes

including cancer, has come to be appreciated as an ideal way to eliminate cancer cells. Apoptosis is a genetically programmed process of cell death required for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli. Cells committed to apoptosis are characterized by membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation (Wyllie *et al.,* 1980). Tremendous progress has been made in understanding apoptosis as a result of the molecular identification of the key components of this intracellular suicide program. (Rathmell *et al.,* 1999; Sellers *et al.,* 1999) There are currently two well-characterized caspase-activating cascades that regulate apoptosis.

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane. In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell. In the extrinsic pathway, signal molecules known as ligands, which are released by other cells, bind to transmembrane death receptors on the target cell to induce apoptosis. For example, the immune system's natural killer cells possess the Fas ligand (FasL) on their surface (Csipo *et al.,* 1998). The binding of the FasL to Fas receptors (a death receptor) on the target cell will trigger multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. Active caspase-8 can also cleave BID protein to tBID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome C in the intrinsic pathway (Adrain *et al.,* 2002).

The intrinsic pathway is triggered by cellular stress, specifically mitochondrial

stress caused by factors such as DNA damage and heat shock (Adrain *et al.*, 2002). The mitochondrial phase that follows eventuates in the release of cytochrome C and the consequent activation of caspases, enzymes in which action leads to the variety of phenotypic alterations characteristic of apoptotic death. Upon receiving the stress signal, the proapoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. However, the signal of BAX and BID is not enough to trigger a full release. BAK, another proapoptotic protein that resides within the mitochondria, is also needed to fully promote the release of cytochrome C and the intramembrane content from the mitochondria (Hague *et al.*, 2004). Following the release, cytochrome C forms a complex in the cytoplasm with adenosine triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme. Following its formation, the complex will activate caspase-9, an apoptosis initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome C, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation.

3. Involvement of p53 in intrinsic pathway of apoptosis

Cancer is associated with decreased apoptosis, and development of apoptosis resistance in cancer cells is a significant contributing factor to the failure of cancer therapies. Thus, induction of apoptosis in apoptosis-resistant cancer cells through a variety of approaches would be an ideal strategy for effective cancer therapy (Reed *et al.*, 2003). One promising approach to achieve this end is through the modulation of p53 or the components of p53 signaling pathways. Often, p53 is referred to as the "guardian of the genome" (Levine, 1997). In response to DNA damage and other types of stress, p53 is able to induce cell growth arrest, apoptosis, and cell senescence (Prives *et al.*, 1999). Mutations within the p53 tumor suppressor gene have been well documented in >50% of all human tumors (Hollstein *et al.*, 1994). In cells that retain wild-type p53, multiple regulatory pathways play an important role in modulating its activities in vivo (Lohrum *et al.*,

1999). Further, p53 promotes tumor suppression through its ability to bind specific DNA sequences and to act as a transcription factor (El-Deiry *et al.,* 1993).

It is generally thought that the mechanism of p53 activation by cellular stress involves mainly posttranslational modifications, including phosphorylation and acetylation (Appella *et al.,* 2000). The importance of p53-mediated transcriptional activation is underscored by the fact that the vast majority of tumor-associated p53 mutations occur within the domain responsible for sequence-specific DNA binding (El-Deiry *et al.,* 1993). The p53 gene is tightly regulated, with the protein often found in latent form and the protein levels being very low in unstressed cells (Freedman *et al.,* 1999). This regulation is essential for its effect on tumorigenesis, as well as for maintaining normal cell growth. Due to its pivotal role in controlling abnormal cell growth and its frequent inactivation in a majority of human cancers, p53 has been a central cancer target for mechanism-driven novel cancer drug discovery. Several different approaches aiming at p53 reactivation or mutant p53 elimination have been attempted in recent years. One approach is to restore wild-type p53 conformation in mutant p53-containing cancer cells by small molecule compounds that have captured a great interest in the field of chemotherapy. The downstream events mediated by p53 take place by two major pathways: cell cycle arrest and apoptosis. Moreover, a number of genes that are critically involved in either cell growth arrest or apoptosis have been identified as p53 direct targets. These include p21, MDM2, and PUMA, among others (El-Deiry *et al.,* 1993; Nakano *et al.,* 2001; Okamoto *et al.,* 1994) The MDM2 oncogene encodes an inhibitor of the p53 tumor suppressor protein that regulates p53 in a negative feedback loop. An important function of MDM2 is to bind to the p53 tumor suppressor protein, inhibiting its ability to act as a transcription factor (Momand *et al.,* 1992). Therefore, the MDM2 negative feedback pathway is an important limiting factor in DNA damage-induced p53 activation. MDM2 gene amplification and over expression occur in several types of tumors and are often associated with poor prognosis. Inhibition of MDM2 is associated with a decrease in MDM2-p53 complex formation, increase in p53-inducible gene expression,

increase in p53 transcriptional activity, and apoptosis. Significantly, inhibition of MDM2 expression enhances the activation of p53 by a DNA-damaging cancer chemotherapy agent in a synergistic fashion. Further, PUMA (p53 up-regulated modulator of apoptosis) can be directly activated by p53 through p53-responsive elements in its promoter region. A number of chemotherapeutic agents have shown to be involved in induction of PUMA in a p53-dependent fashion.

Expression of p53 further induces transcriptional activation of p21, which inhibits cdk-mediated RB (retino-blastoma) protein phosphorylation and functioning of E2F, leading cells to arrest in G_1 (Hartwell and Kastan, 1994). The product of the retinoblastoma susceptibility gene, Rb, plays a central role in the G_1/S transition. In its un- or hypophosphorylated state, Rb prevents progression from G1 to S phase through its interaction with members of the E2F transcription factor family. This interaction not only blocks transcriptional activation by E2F but also actively represses transcription by recruiting histone deacetylase to the promoters of genes required for S phase entry. During cell cycle progression, Rb is inactivated by phosphorylation, which occurs through the sequential actions of D-type cyclins, acting with cdks 4 and 6, and of cyclin E-cdk2 complexes. In response to mitogenic activation, cells synthesize D-type cyclins. The assembly of these proteins with cdks 4 and 6 requires a member of the Cip/Kip family of proteins including p21. They act in stoichiometric amounts to promote the activity of cyclin D-dependent kinases, and they also serve as potent inhibitors of cdk2. Therefore, cyclin-dependent kinases facilitate G_1 progression in two ways. First, they participate in Rb phosphorylation, which relieves transcriptional repression by the Rb-E2F complex (Harbour *et al*., 1999). Second, they sequester Cip/Kip proteins, which facilitate the activation of cyclin E/cdk2 (Sherr and Roberts, 1999). Cyclin E/cdk2–mediated Rb phosphorylation disrupts the binding of Rb to E2F (Harbour et al., 1999), allowing E2F activation and the transcription of genes necessary for S phase entry and progression. While Rb is the primary target of cyclin D–dependent kinases, cyclin E-cdk2 phosphorylates other targets as well, and, even in Rb-deficient cells, this complex is indispensable for S phase entry. In

a Transfection experiments have shown that over-expression of E2F or cdks promotes S phase entry, whereas over-expression of the RB family of proteins arrests cells in the G_1 phase of the cell cycle (Zhu, 1993; Nevins *et al.,* 1994 Dyson, 1998) suggests that the effects of pRB family proteins on cell proliferation are mediated by their repressive effects on E2F-mediated gene expression.

Recent evidence suggests that intracellular signals governing cell proliferation and cell cycle progression also mediate apoptosis (Lee *et al*., 1993). Also, p53 activates expression of Bax (a cell death inducer) and suppresses Bcl-2 (a cell death inhibitor), leading to cellular apoptosis (Reed, 1994). Bax is a proapoptotic protein whose expression is regulated by p53 (Miyashita and Reed, 1995). In turn, Bax acts on mitochondria to cause the release of cytochrome C (Narita *et al*., 1998). Bax moves from cytosol to the mitochondria under conditions that induce cell death by apoptosis. Although the mechanism that mediates this translocation of Bax is not fully understood, evidence suggests that it is a consequence of a conformational alteration in the protein as a result of changes in the ionic composition of the cytosolic milieu (Khaled *et al*., 1999).

4. Matrix metalloproteinases (MMPs) and NF-κB: Other main targets in anticancer therapy.

Presently, clinicians and researchers are focusing more on the concept of targeted therapies and the finding of new and relevant prognostic factors to better delineate treatment options in cancer patients. Clinicians use a number of factors to stratify cancer patients at diagnosis, in order to accurately define risk profiles and plan the most appropriate treatment. Along with patient and tumor characteristics (such as age at diagnosis, tumor size, and lymph node status), an increasing number of molecular prognostic factors, (named tumor "biomarkers"), are being developed for use in patient outcome and treatment determinations [Armstrong and Taylor, 2005]. These markers must have some defining characteristics, such as ease of specimen collection and a reproducible assay that is rapid and inexpensive (Nelson *et al*., 2000). Although hundreds of these

biomarkers have been reported, few are proving to be useful in the clinic. Identification of the importance of proteinases in tumor invasion, a particular interest in a group of enzymes called matrix metalloproteinases (MMP) was developed in recent years to control cancer metastasis. Large amounts of data have been reported relative to MMP over expression in various tumor types when compared to normal tissues (Agnantis *et al*., 2004; Davidson *et al*., 1998; Kugler *et al*., 1998; Hashimoto *et al*., 1998; Sutinen *et al.*, 1998; Gonzalez-Avila *et al*.,1998; Nawrocki *et al*., 1997; Bramhall *et al*., 1997), and several studies have provided evidence that certain MMPs in specific cancers can be useful as indicators of disease progression, thereby improving treatment strategies and management of specific cancers (Mannello *et al*., 2005).

Further, the transcription factor NF-κB was recently identified as another plausible target to treat cancer. The transcription factor NF-κB is well established as a regulator of genes encoding cytokines, cytokine receptors, and cell adhesion molecules that drive immune and inflammatory responses (Sen and Baltimore, 1986). In a number of recent studies, NF-κB activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, the cell cycle, differentiation, and cell migration (Gilmore *et al*., 1996; Bargou *et al*., 1997; Mayo *et al*., 1997; Rayet *et al*., 1999; Madrid *et al*., 2000). Additionally, activation of NF-κB in cancer cells by chemotherapy or by radiation can blunt the ability of the cancer therapy to induce cell death. Compelling evidence support the notion that NF-κB is dysregulated in many forms of cancer and that its inhibition is a logical therapy for certain cancers and for adjuvant approaches to cancer therapy.

5. Chemotherapy as means of treating cancer

Chemoprevention, which refers to the administration of synthetic or naturally occurring agents to prevent the initiation and/or promotional events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasia. The possible role of chemotherapy in treating

illness was discovered when the bone marrow suppressive effect of nitrogen mustard was noted in the early 1900's. Since that time, the search for drugs with anticancer activity has continued, and the goal of treatment with chemotherapy has evolved from relief of symptoms to cure. The ability of chemotherapeutic agents to inhibit cancer cell growth and to initiate apoptosis is an important determinant of their therapeutic response. Many reported agents exert antitumor effects in various types of human cancers cell lines through the induction of apoptosis and cell-cycle arrest. Thus, chemo-preventive agents that can modulate apoptosis may be able to affect the steady-state cell population, which may be useful in the management and therapy of cancer. In recent years, many studies have shown an association of cell cycle regulation and apoptosis with cancer, in as much as the cell cycle inhibitors and apoptosis-inducing agents are being appreciated as weapons for the management of cancer (Ahmad *et al*., 1997; Ahmed *et al*., 2000; Deigner *et al*., 1999; Evan *et al*., 1998; Stadler *et al*., 2000). Each drug varies in the way this occurs within the cell cycle. The major categories of chemotherapy agents are alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, and steroid hormones. Each drug is categorized according to their effect on the cell cycle and cell chemistry.

Most of these anticancer chemotherapeutic agents act non-specifically by exerting damage to DNA. Also development of undesirable side effects has raised the necessity of identification of novel anticancer agents having better effects. In this context, great interest is continually growing to identify and develop effective novel anticancer drugs. Some of identified anti-cancer drugs kill susceptible cells through specifically via induction of apoptosis. Therefore, it appears that differences in the apoptotic pathways which lead to apoptotic deficiency may account for the ability of some tumor cells to resist drug therapy.

6. Drug resistance in melanoma

Human melanoma, a malignancy which develops through the malignant transformation of melanocytes, is a major medical problem, characterized by

rapidly growing incidence and mortality rate. During the past decade, the incidence of melanoma has increased steadily. Age-adjusted incidence rates are about 6 to 10 per 100,000 in Germany, 12 per 100,000 in the United States and 3-fold higher in some geographic areas, such as Australia (Ross *et al*., 1991). Chemotherapy is an extremely ineffective and unsatisfactory means of treating malignant melanoma due to the drug-resistance characteristic of this disease, which either is intrinsic at onset or develops during application of cytostatic drugs (Garbe, 1993) The underlying cellular resistance mechanisms involved in the chemoresistance of melanoma have not been clarified (Serrone, 1999) Alternative treatment methods based on immunological principles are presently being developed but have not reached the stage where they could be applied routinely in clinical practice. For this reason, chemotherapy will continue to be the primary treatment method.

The complexity of the molecular variants involved in signal transduction along apoptotic pathways suggests that the cell may have a variety of possibilities for regulating apoptosis and generating apoptotic deficiency. The inability of melanoma to undergo apoptosis in response to chemotherapy and other external stimuli that poses a selective advantage for tumor progression, metastasis formation as well as resistance to therapy has been a major study target in recent studies (Soengas and Lowe, 2003). Recently, Apaf-1, known to be critically involved in the mitochondrial apoptotic pathway, was described as being frequently down-regulated in melanoma primary and metastatic cell lines (Soengas *et al.,* 2001; Soengas *et al.,* 2003), which was suggested as a therapeutic tool in melanoma treatment. Apaf-1 is a novel 130 kDa cell-death effector protein that acts with cytochrome C and caspase-9 to mediate p53 dependent apoptosis. Apaf-1 contains an N-terminal caspase recruitment domain, which is responsible for recruiting caspase-9, a nucleotide-binding oligomerization domain, and 13 WD40 repeats, which are thought to interact with cytochrome C (Riedl *et al.,* 20050. A negative correlation of Apaf-1 to malignancy, drug resistance, and tumor progression in melanoma and other tumors has also been shown by other groups

(Jia *et al.*, 2001; Fu *et al.*, 2003; Baldi *et al.*, 2004). In addition, a higher frequency of allelic imbalance of the Apaf- 1 locus in metastatic compared with primary melanoma was discovered (Watanabe *et al.*, 2003; Fujimoto *et al.*, 2004).

There are a number of drugs that have been suggested as drug candidates to treat melanoma. However most of these drugs have not confirmed their ability to successfully control the malignant melanoma. Therefore a great interest exists in present world to develop specific anticancer agents to control melanoma.

7. Potential of nutraceuticals to prevent cancer

The importance of nutraceuticals in cancer prevention and treatment remains largely under-exploited, despite increasing evidence showing that these molecules have both chemo-preventive and chemo therapeutic ability. Notwithstanding, the considerable progress made in the design of novel anti-cancer drugs in recent years, one clear lesson from the recent decades of research into cancer is that, although we can treat cancer and induce remission, survival rates have changed little in most cancers (Armstrong *et al*., 2005). Moreover, most anticancer drugs have several toxic side-effects that may produce a poor quality of life for cancer patients and considerable cost in supporting care (Coussens *et al*., 2002). One could conclude that the chances of developing effective therapies would greatly increase with improved knowledge of the progression of specific cancer types and stages with the appropriate tools for evaluating activity at both the molecular and clinical levels. What remains clear is that there is still much basic and translational groundwork to be done to develop and validate tools identifying tumor expressing target enzymes and, primarily, to assess the efficacy of specific compounds (and optimal doses) that significantly limit tumor-associated proteolytic activity (Mannello *et al*., 2005), but clinical research needs to focus on improving the design of trials that better assess natural agents with tumoricidal activity. Nutraceuticals or natural biodrugs may offer several advantages as anti-cancer products because these diet-derived compounds are nontoxic, widely available and inexpensive. Their activity appears to act through multiple targets and

suggesting the presence of several natural components that could synergistically act together to control tumor growth and neovascularization. It is clear that anti-cancer biodrugs designed by nature and used for several thousands of years with little toxicity may prove useful in both treating and preventing cancer.

It is suggested that positively charged functional groups in some macromolecules such as chitosan (parent molecule of the glucosamine) interact with the negatively charged molecules in cancer cell surface and induce specific signaling cascades (Ronghua *et al.,* 2006). More specifically, it is proposed that polycationic nature and proper molecular weight of chitooligosaccharides greatly contribute to its anticancer activities (Muzzarelli, 1977; Caiqin *et al.*, 2002). However, due to higher molecular weights these polymers are not expected to incorporate into cells. Recently, it was reported that, some small and positively charged molecules could selectively target the mitochondria in cancer cells due to their unique negative charge compared to mitochondria in normal cells (Valeria *et al.,* 2002).However, many of the studies that have dealt with such molecules have not come up with the exact mechanism of action revealing target molecules that affect following treatment.

There have been a large number of nutraceutical innovations of glucosamine (Glc) supplements in the present world. Commercially, most Glc is obtained from food-processing wastes (crab, shrimp, or lobster), and the recent emergence of higher valued nutraceutical market for Glc has stimulated worldwide production. However, Glc is also produced naturally virtually by all cells (Rossetti, 2000; Hart *et al.,* 1995; Uldry *et al.,* 2002). An increasing interest is developing in the current world to use Glc as a food supplement or as nutraceutical due to their suggested beneficial roles in the human body. In 1953, Quastel and Cantero (1953) demonstrated that Glc possesses tumor-inhibiting activity. Since then, a number of reports have confirmed the tumoricidal activity of Glc (Bekesi *et al.,* 1970; Fare *et el.,* 1967). Recent reports suggested a role of Glc in immunosupression (Ma *et al.,* 2002), modulation of expression of inflammatory enzymes (Yudoh *et al.,* 2005), suppression of activation of T-lymphoblasts and dendritic cells (Ma *et al.,* 2002).

Glc also has been shown to inhibit nucleic acid and protein biosynthesis and irreversible damage to organelles in tumor cells, but not in normal cells (Molnar *et al.,* 1972). In addition, Glc also inhibits platelet aggregation and ATP release induced by *Staphylococcus aureus*, ADP, epinephrine, and collagen (Bertram *et al.,* 1981). The mechanisms by which Glc acts are not completely clear; however, it has been shown to alter the ultrastructure of plasma and intracellular membranes (Molnar *et al.,* 1972), to inhibit membrane transport of nucleosides (Friedman *et al.,* 1977), and reportedly to shift its distribution from glycoproteins to glycolipids (friedman *et al.,* 1980).

Glucosamine is available commercially as a nutritional supplement in three forms: glucosamine hydrochloride, glucosamine sulfate and N-acetyl-glucosamine. At neutral as well as physiologic pH, the amino group in Glc is protonated, resulting in its having a positive charge. Salt forms of Glc contain negative anions to neutralize the charge. In glucosamine hydrochloride, the anion is chloride, and in glucosamine sulfate the anion is sulfate. N-acetylglucosamine is a delivery form of Glc in which the amino group is acetylated, thus neutralizing its charge. To date, most of the clinical studies examining the effect of Glc on osteoarthritis have been performed with either the sulfate or the chloride salts of Glc. The characteristic of Glc that is most important in the field of chemistry is this monomer has nucleophilic primary amino group. The reactivity of this amino group is convenient as they allow Glc derivatives to be generated with a range of facile chemistries or biochemistries.

In the present research quaternized amino functionality was substituted to Glc to obtain a strong cationic molecule having smaller molecular weight. This novel and positively charged Glc derivative having smaller molecular weight was expected to exert better effects as ab anticancer agent. And this present study was designed to carryout a detailed mechanistic evaluation of this novel positively charged Glc derivative having quaternized amino functionality.

Materials and Methods

1. Materials

Chitooligosaccharides were kindly donated by Kitto Life Co. (Seoul, South Korea). Chemicals required for synthesis, including 2, 3-epoxypropyl chloride and tri-methyl amine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the cell lines used in this study were purchased from American Type of Culture Collection (Manassas, VA, USA). Materials required for culturing of cells including culture media were purchased from Gibco BRL, Life Technologies (USA). Tunel assay kit was purchased from Upstate cell signaling solutions (Lake Placid, NY). Trizol® was obtained from Invitrogen (CA, USA). AMV reverse transcriptase was purchased from USB Corporation (OH, USA). RC-DCTM protein assay kit was obtained from Bio-Rad (CA, USA). Dowex-50WX2 cation exchange resin was purchased from Dow Chemical Company (Michigan, USA. Dialysis membrane having 100 Da pore size was purchased from Spectrum Laboratories Inc. (RanchonDominguez, CA). Annexin V was obtained from MolecularProbes (OR, USA). MTT reagent, propidium iodide, RNase, Proteinase K, ethidium bromide and dithiothreitol (DTT) were also purchased from Sigma Chemical Co. (St. Louis, MO, USA). p53, p21, NF-κB and AP-1 gene promoter reporter vectors were purchased from Clontech (CA, USA). MMP-9 and MMP-2 gene promoter reporter vectors were kindly donated by Dr. Sang-Oh Yoon (KAIST, Taejeon, Korea).

2. Instrumental analysis

Infrared spectra were recorded using KBr plates in Spectrum 2000® FT-IR spectrophotometer (Perkin Elmer, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded in a D_2O environment using a JNM-ECP-400® (400 MHz) spectrometer (JEOL, Japan). Elemental analysis (C, N and H) was performed using a Vario-EL Elementar Analysesysteme® (USA) and optical density was measured using GENios® microplate reader (Tecan Austria GmbH, Austria). Fluorescence emitted

from PI and Annexin-V was measured at FL2 (590 nm), and 10,000 events/sample were acquired using XL-MCL™ flow cytometer equipped with EXPO™ 32 software (Beckman Coulter, Inc., CA, USA). Fluorescent microscope (Axiovert 200, Zeiss.) with Axio vision 3.1 software was used to quantify fragmented DNA bound with fluorescence emmiting substrate. To quantify protein expression levels, western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

3. Preparation of glucosamine (Glc) from chitosan

Chitooligosaccharides (10.0 g, average deacetylation degree of 90-97%) were mixed with 50 ml of 6 N HCl and stirred at 100 ºC while refluxing. After 3 h, 50 ml of distilled water and 1.0 g of activated carbon powder were added and stirring was continued for additional 30 min at 60 ºC. Reaction mixture was then filtered using a glass filter and addition of activated carbon powder (1.0 g) to the filtrate was repeated to obtain a clear solution. This solution was then vacuum evaporated and the resulting solid was thoroughly washed with ethanol and diethyl ether to give Glc (8.2 g).

Glc: fine white solid; IR (KBr) ν_{max} 3412 (OH), 2864 (CH), 1578 (NH), 1309 (CN), 1108, 1065, 1036 (pyranose) cm^{-1}; ^{1}H NMR (D_2O, 400 MHz) δ 5.4, 4.9 (1H, H-1_α, H-1_β), 3.2, 2.9 (1H, H-2_α, H-2_β), 3.4 ~3.9 (1H, H-3, 1H, H-4, 1H, H-5 and 2H, H_2-6), 4.7 (D_2O); ^{13}C NMR (D_2O, 400 MHz) δ 89.0, 92.1 (C-1_α, C-1_β), 54.3, 56.2 (C-2_α, C-2_β), 69.0 (C-3), 76.3 (C-4), 71.2, 72.4 (C-5_α, C-5_β), 61.1 (C-6); elemental analysis C% (33.48), N% (6.49), H% (6.61).

4. Synthesis and purification of quaternized amino glucosamine (QAGlc)

Glc was quaternized by reacting with 2,3-epoxypropyl chloride and trimethylamine according to the method of Ronghua *et al.* (2003). For the synthesis, pH of the trimethylamine solution (1.58 ml) was first adjusted to 2.0 by 1N HCl. Same molar ratio 2,3-epoxypropyl chloride (0.8 ml) was added drop wise to trimethylamine solution, following adjustment of the pH of trimethylamine

solution to 9.00 by 1M NaOH. Glc (2.15 g) was then added at same molar ratio to the reaction mixture containing trimethylamine and 2,3-epoxypropyl chloride. Then the quarternization reaction was preceded at room temperature while stirring for 24 h. Throughout the reaction, pH of the reaction mixture was maintained at 9.0 using 1M NaOH. Reaction product was extracted using ethanol, methanol and trihydrofluoride and finally quaternized amino glucosamine (QAGlc) was obtained (1.4 g). Then QAGlc (1.4 g) was subjected to dialysis using 100 Da molecular weight cut-off membrane to remove un-reacted 2,3-epoxypropyl chloride and trimethylamine. After series of dialysis attempts, lyophilized QAGlc (1.2 g) was passed through a Dowex-50WX2 cation exchange resin and amount of carbohydrate in fractions eluted with 1 N NaCl was measured using modified phenol-sulfuric acid method of Fox and Robyt (1990) by measuring the absorbance at 420 nm. After plotting the graph, fractions were pooled and QAGlc was separated as a solution from un-reacted Glc. QAGlc solution was further subjected to dialysis using a 100 Da molecular weight cut-off dialysis membrane and lyophilized to give QAGlc (1.05 g).

QAGlc: dark brown fluffy solid; IR (KBr) ν_{max} 3411 (OH), 2929, 2805 (CH), 1639 (CO), 1480 (Me), 1309 (CN), 1115, 1092, 1033 (pyranose) cm^{-1}; ^{1}H NMR (D_2O, 400 MHz) δ 5.4, 4.9 (1H, H-1_α, H-1_β), 3.2, 2.9 (1H, H-2_α, H-2_β), 3.4 ~3.9 (1H, H-3, 1H, H-4, 1H, H-5 and 2H, H_2-6), 4.7 (D_2O), 2.8 (2H, H_2-7 and 2H, H_2-9), 3.1 (9H, H_9-10); ^{13}C NMR (D_2O, 400 MHz) δ 89.2, 92.4 (C-1_α, C-1_β), 54.5, 56.1 (C-2_α, C-2_β), 69.3 (C-3), 76.1 (C-4), 71.4, 72.3 (C-5_α, C-5_β), 61.3 (C-6), 64.4 (C-7), 57.2 (C-8), 65.1 (C-9), 54.2 (3C,10-NMe); elemental analysis C% (43.61), N% (8.52), H% (8.19).

5. Cell culture

DMEM F-12 medium for normal human dermal fibrpblasts (HDF), MRC5, B16F1, MG63, SW1353, Hela, A549, SW480 cell lines, DMEM medium for RAW264.7, HT1080 cells and RPMI medium for U937 cells were used to culture cells with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin-

streptomycin in 10 cm^3 culture dishes. For experiments, cells were detached with trypsin-EDTA and plated into 24- or 96-well plates at a plating density of 7×10^5 and 1.5×10^5 per well, separately.

6. Toxicity determination of QAGlc on cancer cells derived from different tisues

To assess cell cytotoxicity levels, QAGLc was treated into 9 different cancer cell lines and 2 types of normal cells. Following treatment of test compounds, MTT assay was performed using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen *et al.* (1989). Briefly, cells were grown in 96-well plates at a density of 5×10^4 cells/well and were treated with different concentrations of test compounds. After 48 h of incubation, cells were added with 100 μl of MTT (5 mg/ml) and incubated for 4 h. Finally, DMSO (100 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control ((OD of non-treatment group − OD of treatment group / OD of non-treatment group ×100)) and dose response curves were developed. The data were expressed as a mean from at least three independent experiments and $P<0.05$ was considered significant.

7. Determination of apoptosis

Four different approaches were used to assess the induction of apoptosis in B16F1 cells following treatment of QAGlc for different time periods.

7.1. DNA fragmentation assay

DNA fragmentation assay was performed with slight modifications as described by Abid-Essefi *et al.* (2003). Briefly, B16F1 cells (10^7 cells/ml) were grown in 10 cm^3 tissue culture flasks (Nunc, Denmark) for 24 h and determined

concentrations of test compound were added. For comparative purposes, H_2O_2, an apoptosis inducing agent in number of cancer cells was treated at 2 mM concentration in a separate experiment. After incubation for 48 h, the cell layer was rinsed with PBS, trypsinized and the cell pellet was obtained by centrifugation at 1,500 × g for 3 min. The pellet was transferred to an eppendorf tube and lysed in ice for 20 min with 400 µl of cell lysis buffer (10mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100 (pH 8.0). The mixture was centrifuged for 20 min at 13,000 × g (4 ºC) and the supernatant was collected. Phenol-chloroform-isoamylalcohol was added at 1:1 ratio and upper phase was collected after centrifugation at 13,000 × g (4 ºC) for 15 min. Sodium acetate and isopropanol were then added and incubated on ice for 10 min. Following centrifugation the pellet was collected. Pellet was then washed with 70% ethanol and RNase was added to the supernatant to obtain final concentration of 20 µg/ml and incubated for 1 h at 37 ºC. Proteinase K (final concentration of 20 µg/ml) was added to the same medium and incubated for another 1 h at 55 ºC. DNA was dissolved in TE buffer (10mM Tris-HCl pH 8.0, 1m MEDTA) and quantified using UV spectrophotometry at 260 nm. Samples were analyzed by electrophoresis on a 1% agarose gel (1 h at 80 V/30 mA) in the presence of ethidium bromide using Tris-acetate running buffer. Major biochemical hallmark of apoptotic cell death is the cleavage of chromosomal DNA at inter-nucleosomal sites into fragments or multiples of about 200 bp. Therefore, if QAGlc induces apoptosis in melanoma cells, fragmented DNA result from the action of activated endonucleases can be clearly identified.

7.2. Cell cycle analysis for apoptosis determination- PI method

B16F1 cells (1 x 10^6 cells) were treated with 50 µg/ml concentration of QAGlc for different time durations. Propidium iodide staining was used to quantify cells undergoing apoptosis and to analyze cell cycle according to previously reported method (Wiepz *et al.*, 2006). Briefly, treated cells were collected and washed with ice-cold PBS, and fixed with 50% ethanol. Fixed cells

were washed with phosphate buffered saline (PBS) containing 5 mM EDTA and incubated with RNase A at 37 ºC for 30 min. Propidium iodide (PI) with final concentration of 500 ug/ml was added and incubated for 40 min at room temperature in the dark. Fluorescence was measured at FL2 (590 nm), and 10,000 events/sample were acquired using XL-MCL™ flow cytometer equipped with EXPO™ 32 software (Beckman Coulter, Inc., CA, USA).

7.3. Cell cycle analysis for apoptosis determination- Annexin-V method

A combined staining with fluorescein isothiocyanate-conjugated annexin-V and propidium iodide was performed to distinguish normal, apoptotic and necrotic cells according to the method described by Vermes *et al.* (1995) with modifications. Harvested cells were washed once with Annexin-V binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$) and resuspended in 200 µl of the same buffer containing fluorescein isothiocyanate-conjugated annexin-V. After 15-min incubation in the dark at room temperature, cells were diluted with 300 µl of binding buffer, and propidium iodide was added before flow cytometric analysis. Percentage of viable and dead cells were determined by using the FL1 channel for annexin-V and the FL2 channel for PI using XL-MCL™ flow cytometer equipped with EXPO™ 32 software (Beckman Coulter, Inc., CA, USA).

7.4. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

B16F1 cells (1×10^5) culturing in 24 well plates were treated with different concentrations of QAGlc and after 48 h TUNEL assay was employed to identify apoptotic cells having free 3'-OH of cleaved DNA using a kit (TUNEL apoptosis detection kit, Upstate cell signaling solutions, NY, USA) according to manufacturer's instructions. Briefly, treated cells were washed twice with PBS and fixed with 0.5% glutaraldehyde in PBS for 15 min. Following washing cells three times with PBS cells were incubated with a solution containing 0.05% tween,

0.2% BSA in PBS for 15 min at room temperature. For comparative purpose, positive control was prepared by incubating fixed cells with DNase I in PBS for 1 h at 37 ºC followed by washing for 15 min with PBS to terminate the reaction. After washing, free 3'-OH of cleaved DNA in apoptosized cells were labeled by incubating for 60 min with Terminal deoxynucleotidyl transferase (TdT) end labeling cocktail containing Tdt buffer, Biotinylated deoxyuridine triphospahate (Biotin-dUTP) and TdT. Reaction was stopped by adding termination buffer (TB buffer). Cells were then washed with PBS and incubated with the blocking buffer at room temperature for 20 min. following removal of blocking buffer, cells were incubated in dark for 30 min at room temperature with avidin-FITC solution. Since this assay is based on the detection of single and double stranded DNA breaks (nicks) by using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL), localization of fragmented DNA that emit fluorescence can be visualized clearly. Therefore, cells were visualized with fluorescent microscope (Axiovert 200, Zeiss.) using Axio vision 3.1 software and compared with the cells without induction of apoptosis.

8. RT-PCR

Quantitative RT-PCR was used for the analysis of expression of p53 gene using cDNA. Firstly, B16F1 cells were treated with predetermined concentrations of QAGlc for different time durations. A separate experiment was performed by treating cells with etoposide as a positive control to compare effects of QAGLc. Then the cells in different groups were lyzed separately with Trizol®. Chloroform was added to the lysate and centrifuged at 12,000 × g for 15 min and upper layer was collected. Isoporoponol was added at 1:1 ratio and RNA pellet was obtained following centrifugation. After washing with ethanol extracted RNA was solubilized in diethylpyrocarbonate-treated RNase free-water and quantified by measuring absorbance at 260 nm using GENios® microplate reader (Tecan Austria GmbH, Austria). Integrity of RNA was checked by 1% agarose gel elecrophoresis.

For RT-PCR, 1 μg of total RNA was reverse-transcribed to generate first

strand cDNA using AMV reverse transcriptase by reverse transcription (RT) carried out at 42 ºC for 45 min. RT-generated cDNA was used as a template to amplify p53 and G3PDH mRNA in a PCR mixture containing dNTPs, taq DNA polymerase (USB corporation, OH, USA), and specific upstream and downstream primers in 20 mM tris-HCl reaction buffer. For P53, forward primer: 5'-GCTCTGACTGTACCACCATC-3' and reverse primer: 5'-TTCAGCTCTCGGAACATCTC-3' were used. For glyceraldehyde-3-phosphate dehydrogenase (G3PDH) forward primer: 5'-TGAAG GTCGGTGTGAACGGATTTGGC-3' and reverse primer: 3'-CATGTAGGCCATGAGGTCCACCAC-5' were employed. PCR was performed in a Whatman thermocycler (Biometra, UK). The amplified DNA fragments analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The mRNA of G3PDH served as an internal control for sample loading and mRNA integrity.

9. Reporter gene assay

B16F1 cells cultured in 10 cm culture dishes were transiently transfected with P53 promoter containing luciferase reporter vector and p21 promoter containing luciferase reporter plasmid by Lipofectamine™ 2000™ reagent (Invitrogen). Beta-galactosidase expression vector was co-transfected with the reporter vector to serve as an internal control of transfection efficiency. After 24 h of transfection, cells were subcultured into 24 well plates and cells treated with different concentrations of QAGlc for 24 h. Cells were washed once with cold PBS and lysed with 200 μl/well lysis buffer (25 mM Tris-HCl, pH 8.0, containing 2 mM DDT and 1% Triton-X 100). Aliquots of cell lysate and luciferase substrate (Promega) were mixed in equal amounts in a 96-well plate and luminescence intensity was measured with a GENios®luminescence microplate reader (Tecan Austria GmbH, Austria). Beta-galactosidase activity was measured with ONPG buffer and the luciferase activity values were normalized using transfection efficiency monitored by the co-transfected β-galactosidase expression vector. The

level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

Transfection efficiency was determined by X-Gal staining method. Briefly, transfected cells were fixed with 0.5% glutaraldehyde and stained with X-Gal solution containing 20 mM $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and 1 mM $MgCl_2$. After 24 h of incubation at 37°C, transfected cells were visualized with blue color under a light microscope.

10. Western blot analysis

After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Briefly, harvested B16F1 cells were washed twice with ice-cold PBS; resuspended in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) containing antipain (1 μg/ml), aprotinin (1 μg/ml), chymostatin (1 μg/ml), leupeptin (0.1 μg/ml), pepstatin (1 μg/ml), and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and incubated on ice for 20 min. Supernatants were recovered by a 10-min centrifugation (12,000 × *g*) at 4 °C, and protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin as a standard. Proteins (20-40 μg) were diluted in 5x sample buffer (10% SDS and 100 mM each dithiothreitol, glycerol, bromphenol blue, and Tris-HCl) and resolved in 4-20% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane. Then proteins were transferred onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany), and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with the appropriate dilution of primary antibodies (1:500 dilutions) related to apoptosis and cell cycle progression. After three 5-min washes with Tris-buffered saline and 0.1% Tween 20, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. They were then

washed again three times with Tris-buffered saline and 0.1% Tween 20, rinsed briefly with PBS, and developed with enhanced chemiluminescence reagents (ECL, Amersham Biosciences, UK). Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). Detection of β-actin (1:5000 antibody dilutions) was used as control for equal loading of protein.

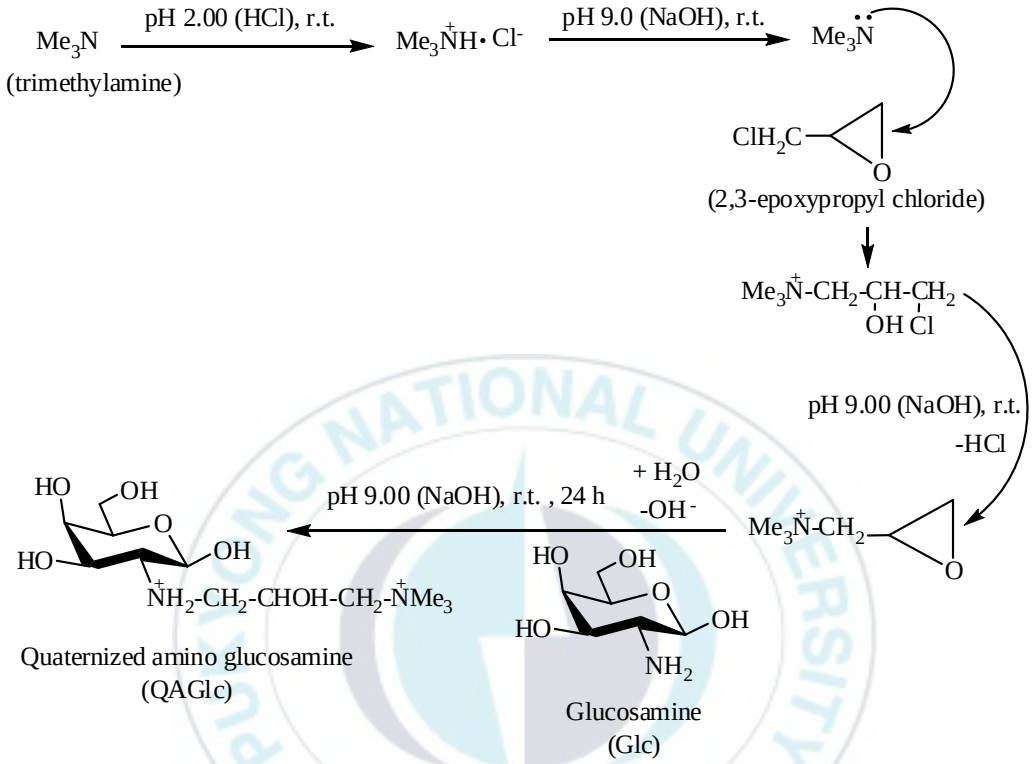
11. Statistical analysis

Results are presented as mean ± standard error of the mean (n = 3). Student's t-test was used to determine the level of significance.

Results and Discussion

1. Synthesis of QAGlc

QAGlc was synthesized following scheme 1. In order to synthesize this Glc derivative having quaternized amino functionality, Glc was reacted with trimethylamine and 2,3-epoxypropyl chloride at same molar ratio. As shown in Scheme 1, adjustment of lower pH in trimethylamine solution was necessary in order to increase the boiling point and it was achieved by acidification to form a salt. When pH is increased to 9.00 by addition of NaOH, the salt is neutralized and become more active to attack 2,3-epoxypropyl chloride. Reaction of trimethylamine with epoxide compounds involves nucleophilic substitution and cleavage of the epoxide ring. The reaction of trimethylamine and 2,3-epoxypropyl chloride gives (2,3-epoxypropyl) trimethylammonium chloride which subsequently reacts with Glc. However, an amino group of the Glc is the most favourable nucleophilic center under alkaline conditions (pH 9) to react with this intermediate. Subsequently, the reaction between (2,3-epoxypropyl) trimethylammonium chloride and amino group of the Glc forms quaternized amino glucosamine. Alkaline pH as well as the addition of trimethylamine, 2,3-epoxypropyl chloride and Glc at same mole ratio is critical for this reaction. After extraction of the product using series of solvents, quaternized amino glucosamine (QAGlc) was obtained as a dark brown fluffy solid. Removal of remaining 2, 3-epoxypropyl chloride and tri-methyl amine was possible by using 100 Da dialysis membrane because both these materials have smaller molecular weights compared to Glc and QAGlc. Following dialysis, lyophilized product which contained unreacted Glc and newly synthesized QAGlc, was separated using a cationic resin at pH 4.0. At this pH, both Glc and QAGlc are adsorbed to cationic resin and they can be eluted at different salt concentrations due to difference in their cationic properties. Finally the purified end product was obtained as a dark brown fluffy solid. This amount represented 50% yield (w/w) of the weight of initial material Glc.



Scheme. 1. Synthesis of QAGlc from Glc.

2. Structural confirmation of QAGlc

In comparison to the FT-IR spectrum of Glc (Fig. 1A), the bend absorption observed at 1480 cm^{-1} (Fig. 1B) that represents the methyl groups in -$CH_2CH(OH)CH_2N^+Me_3$ confirmed the introduction of quaternized amino functionality to Glc. This bend absorption has been used in several other researches that have dealt with substitution of quaternized amino functionality to chitosan and chitooligosaccharides to confirm the success in substitution (Jiyoung *et al*., 2003; Ronghua *et al.,* 2003). As depicted in Fig. 2A & 2B, information from ^{13}C NMR spectra of the Glc and QAGlc supported the substitution of quaternized amino group to Glc. According to these data, the original material, Glc exhibited peaks that represent configurations of C1-C-6. Comparing to Glc, in the spectrum of QAGlc new chemical shifts were observed representing C atoms in -$CH_2CH(OH)CH_2N^+Me_3$ group, at δ 54.2 (N-Me_3), δ 64.4 and δ 65.1 (C-7 and C-9) and δ 57.2 (C-8) respectively. These chemical shifts are in agreement with the NMR data reported in similar studies that has characterized substitution of quaternized amino functionality to chitooligosaccharides (Muzarelli *et al.,* 1982; Jiyoung *et al*., 2003; Ronghua *et al.,* 2006). Further, ^1H NMR spectrum of QAGlc (Fig. 3B) was used to confirm the existence of substituted group compared to ^1H NMR spectrum of Glc (Fig. 3A) and it presented new chemical shifts at δ 3.1 (assigned to protons in N-Me_3), and δ 2.8 (assigned to protons of C-7 and C-9) (Jiyoung et al., 2003; Ronghua *et al.,* 2006). Further, data obtained from elemental analysis strengthened the substitution of -$CH_2CH(OH)CH_2N^+Me_3$ to Glc and analyzed data were in agreement with the calculated elemental composition (Table 1). Taken together, these data confirm the successful substitution of quaternized amino functionality to Glc.

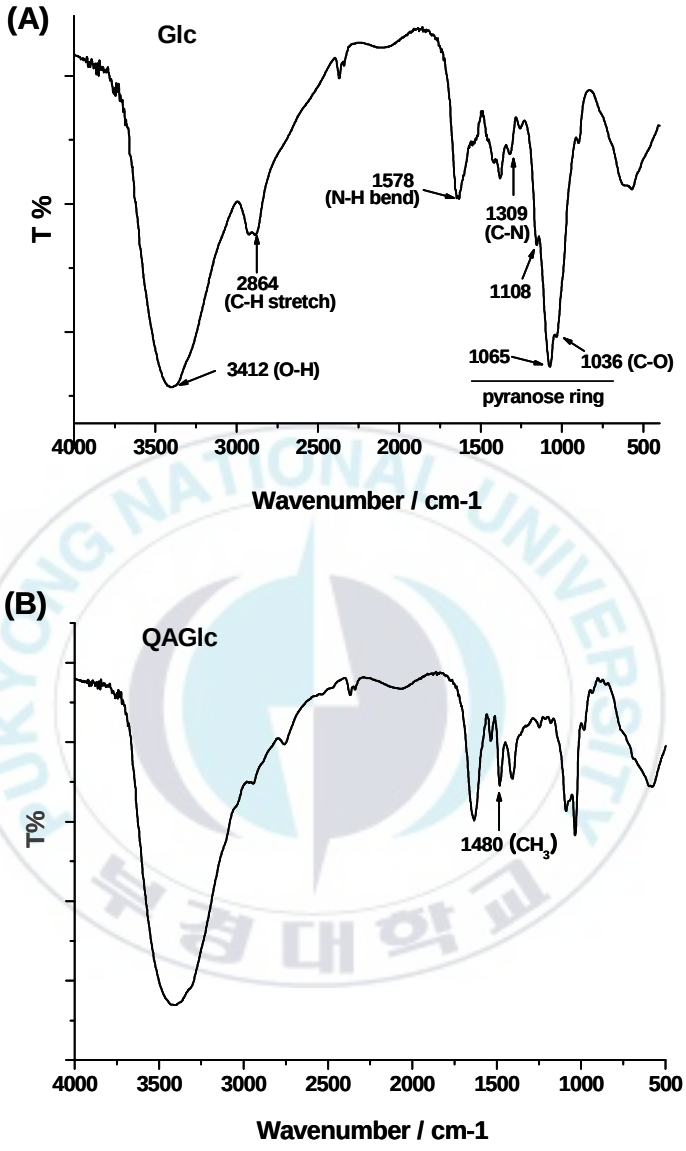


Fig. 1. FT-IR (KBr) spectra of Glc and QAGlc.

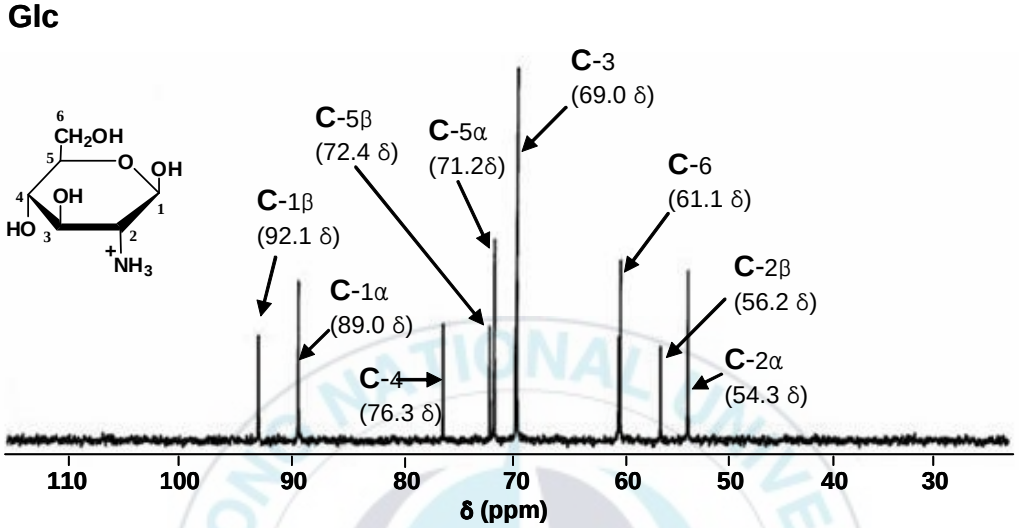


Fig. 2A. ^{13}C NMR (D_2O, 400 MHz) spectrum of Glc.

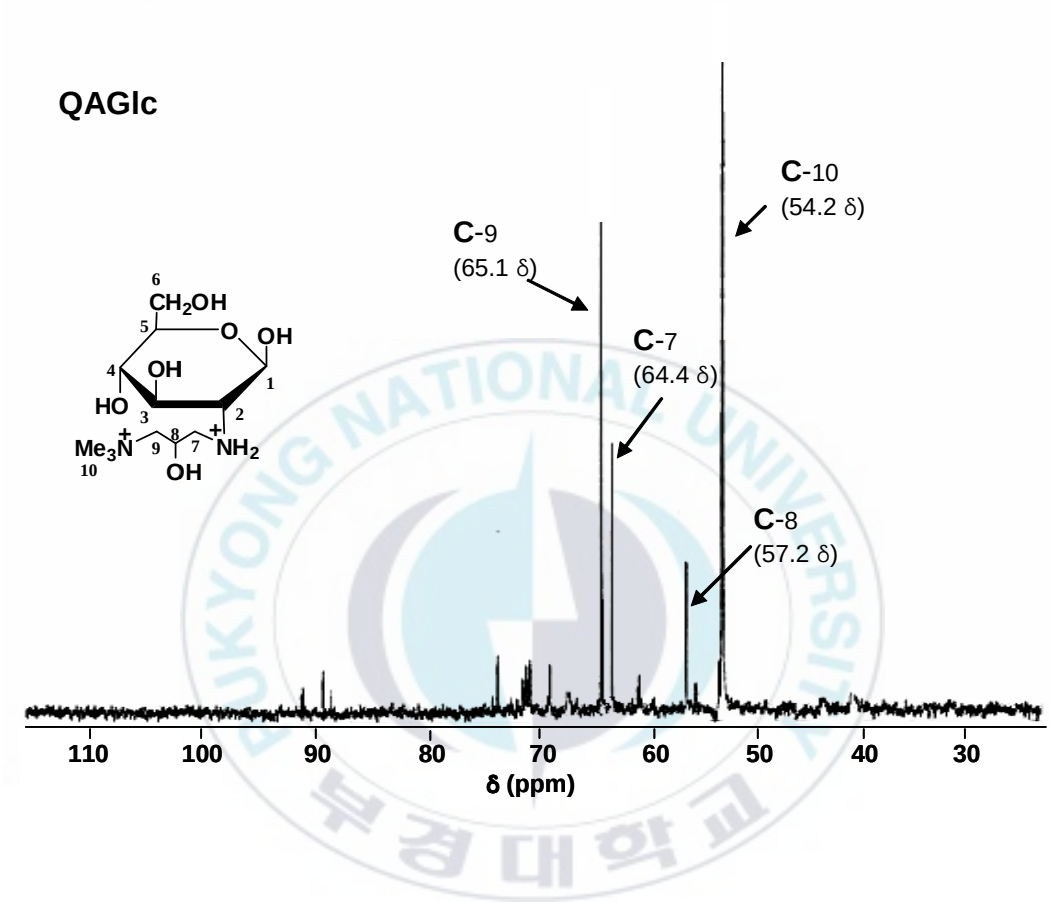


Fig. 2B. ^{13}C NMR (D_2O, 400 MHz) spectrum of QAGlc.

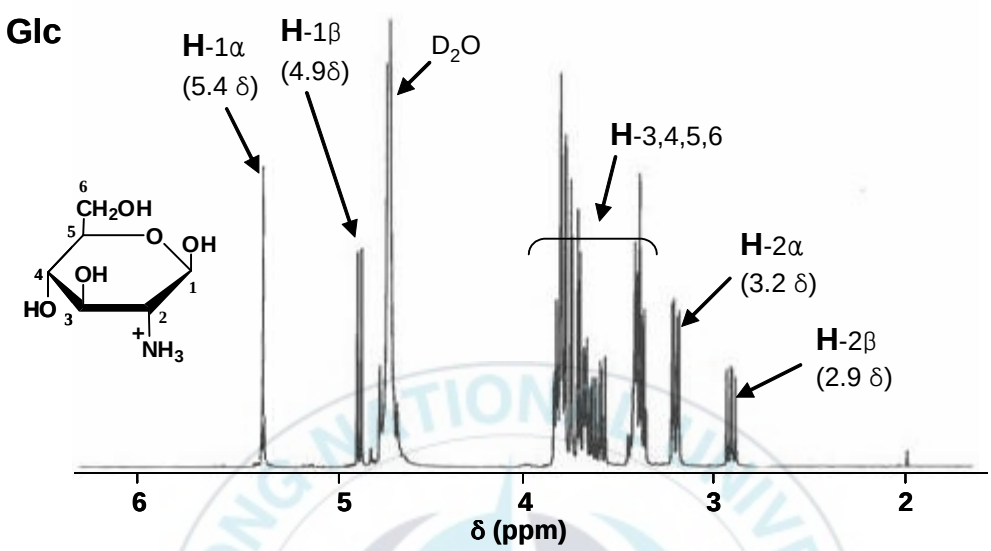


Fig. 3A. ^{1}H NMR (D_2O, 400 MHz) spectrum of Glc.

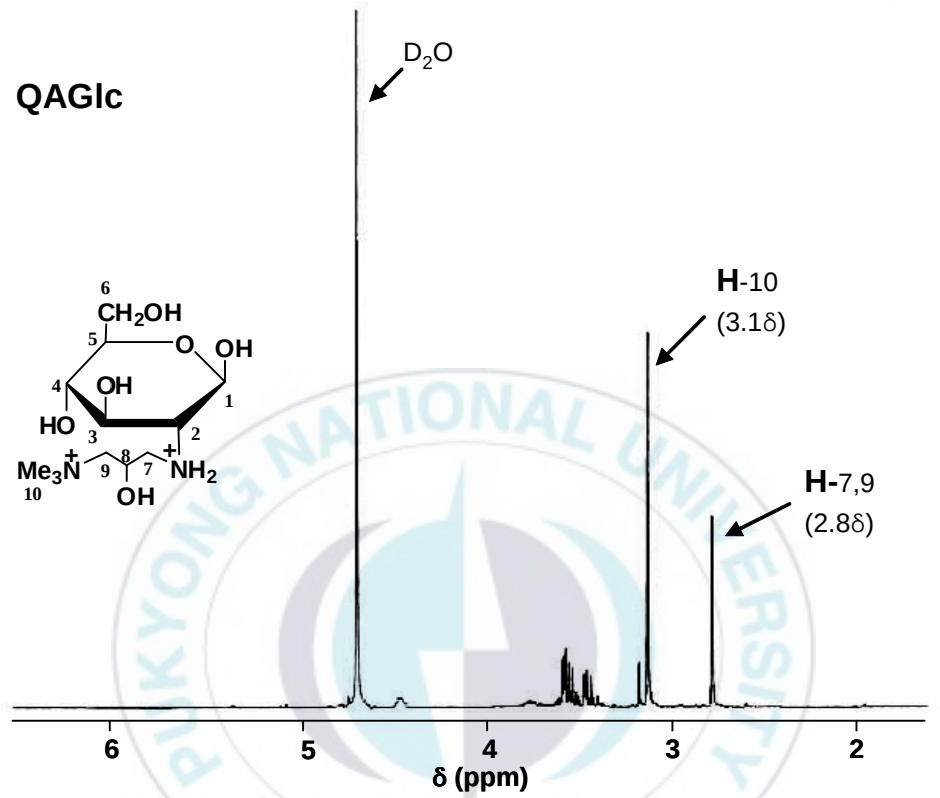


Fig. 3B. ^{1}H NMR (D_2O, 400 MHz) spectrum of QAGlc.

Table 1. Elemental analysis of Glc and QAGlc.

	Carbon content (%)		Hydrogen content (%)		Nitrogen content (%)	
	Anal.	Cal.	Anal.	Cal.	Anal.	Cal.
Glc	33.48	33.42	6.61	6.54	6.49	6.51
QAGlc	43.61	43.57	8.19	8.23	8.52	8.47

3. QAGlc exerted differential effects in cancer cell lines derived from different tissues.

A preliminary screening was performed to assess the effects of QAGlc on proliferation of cancer cells. For this purpose it was treated to nine types of cancer cell lines (Table 2) derived from different tissues and two normal human fibroblast cell lines. However, the toxic effects exerted by QAGlc on normal cell lines as well as on cancer cell lines except B16F1 cells were not significant (Fig. 4, 5, 6, 7, 8).

However, QAGlc exerted higher and clear cytotoxic effect on B16F1 melanoma cells after 48 h of treatment. Therefore time dependent cytoxic effect of QAGlc on B16F1 melanoma cell line was conducted by treating QAGlc at different concentrations, 100 µg/ml, 50 µg/ml, 10 µg/ml and 1 µg/ml (Fig 9). Treatment of B16F1 cells with QAGlc for 12 h did not result in a significant reduction in cell viability even at a higher dose of QAGlc (100 µg/ml), whereas, treatment of QAGlc for 36 h resulted a significant reduction in cell viability. The cell viability was further decreased when cells were incubated for 48 h. Results obtained from MTT assay performed with Glc at similar concentrations and time durations (Fig 10) clearly confirmed that Glc is not cytotoxic to B16F1 cells but the observed cytotoxic effect was due to substitution of quaternized amino functionality to Glc.

Table 2. Cell lines employed to test the cytotoxic effects of QAGlc.

Cell line	Tissue	Disease
MG63	bone	osteosarcoma
U937	blood	histiocytic lymphoma
HepG2	liver	hepatocellular carcinoma
SW480	colon	colorectal adenocarcinoma
A549	lung	carcinoma
HT1080	connective tissue	fibrosarcoma
SW1353	bone	chondrosarcoma
RAW264.7	ascites	abelson murine leukemia virus- induced tumor
B16F1	skin	melanoma
MRC-5	lung	normal
HDF	skin	normal

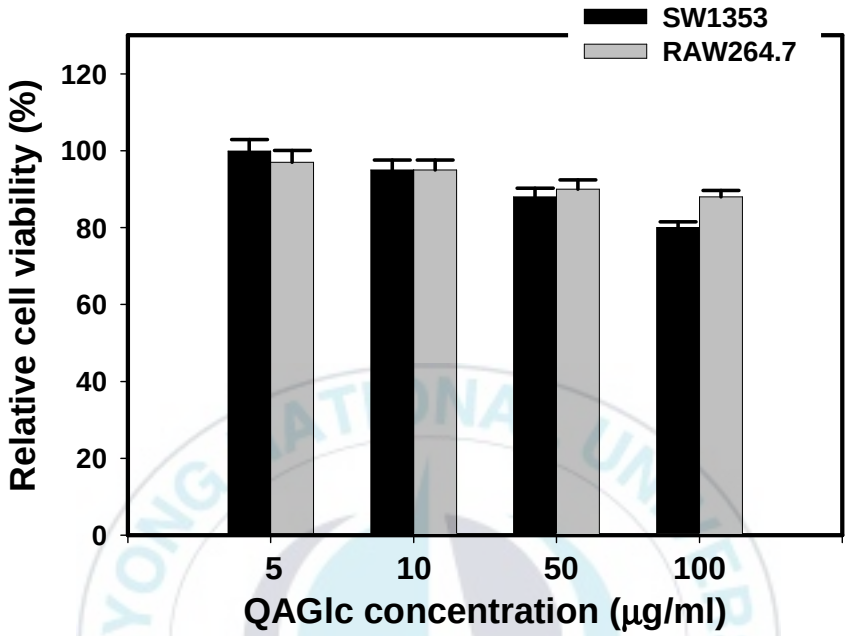


Fig. 4. Cytotoxic effects of QAGlc on SW1353 and RAW264.7 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group / OD of non-treatment group ×100).

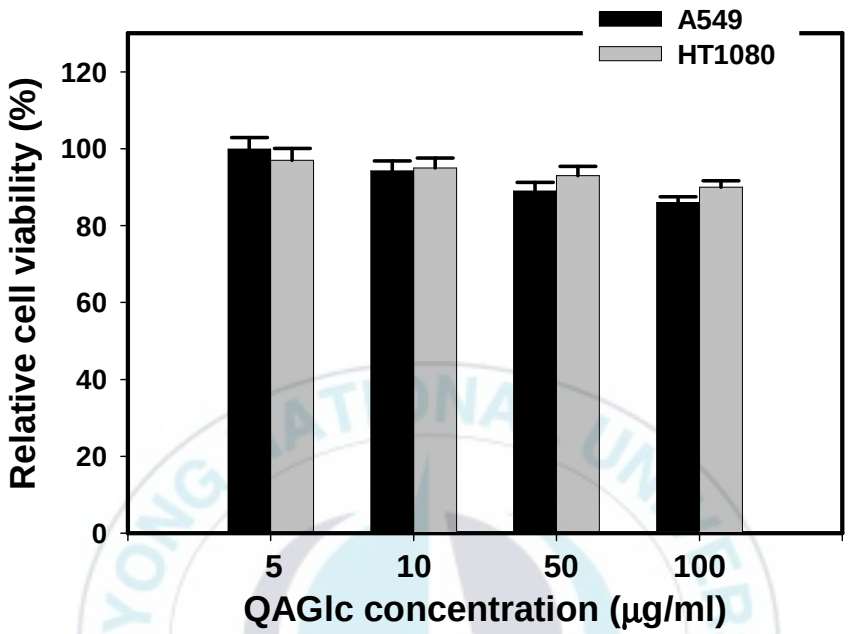


Fig. 5. Cytotoxic effects of QAGlc on A549 and HT1080 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group / OD of non-treatment group ×100).

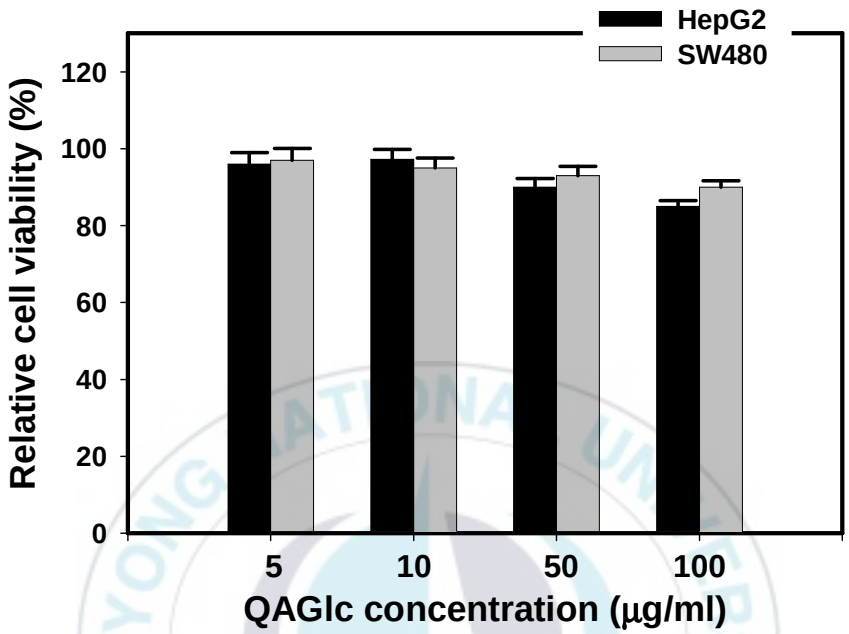


Fig. 6. Cytotoxic effects of QAGlc on HepG2 and SW480 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group / OD of non-treatment group ×100).

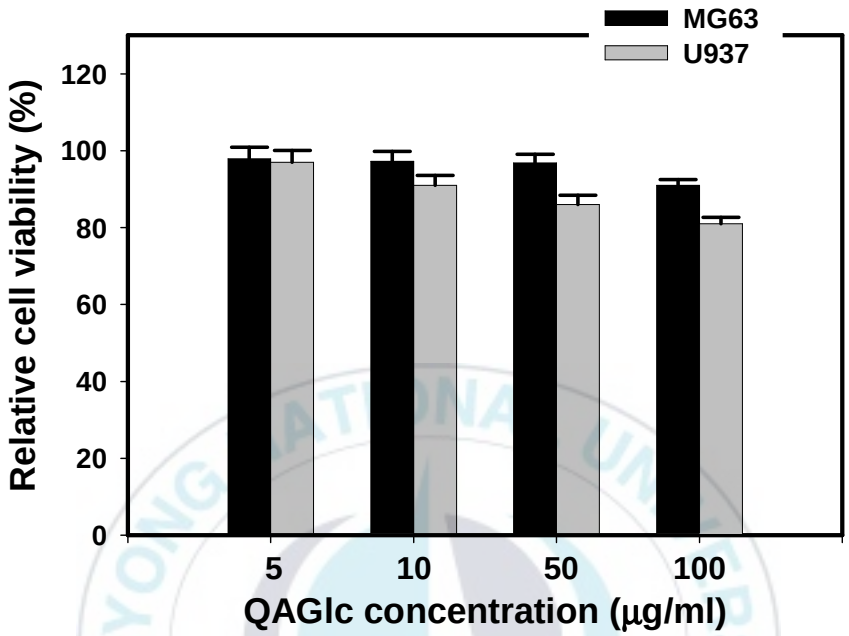


Fig. 7. Cytotoxic effects of QAGlc on MG63 and U937 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group / OD of non-treatment group ×100).

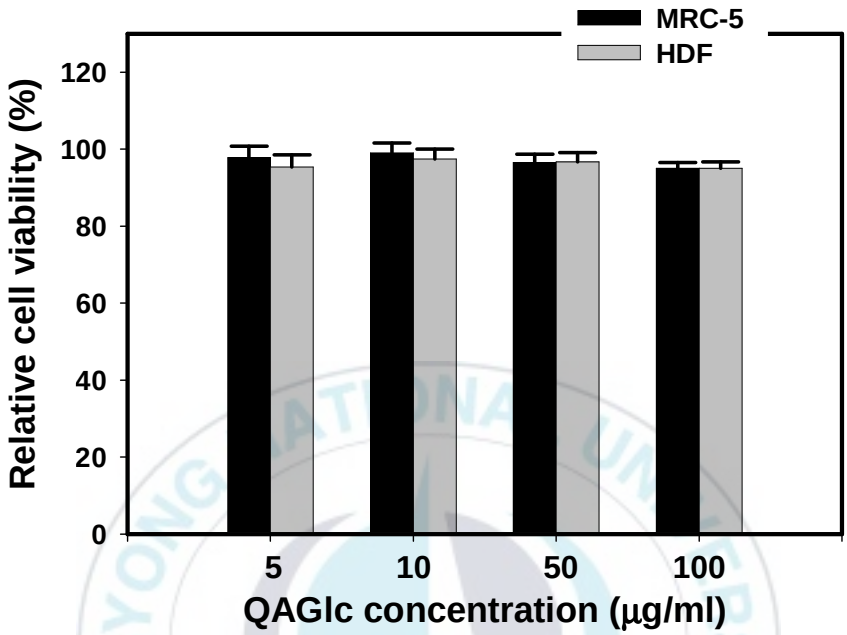


Fig. 8. Cytotoxic effects of QAGlc on MRC-5 and normal human dermal fibroblast (HDF) cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group / OD of non-treatment group ×100).

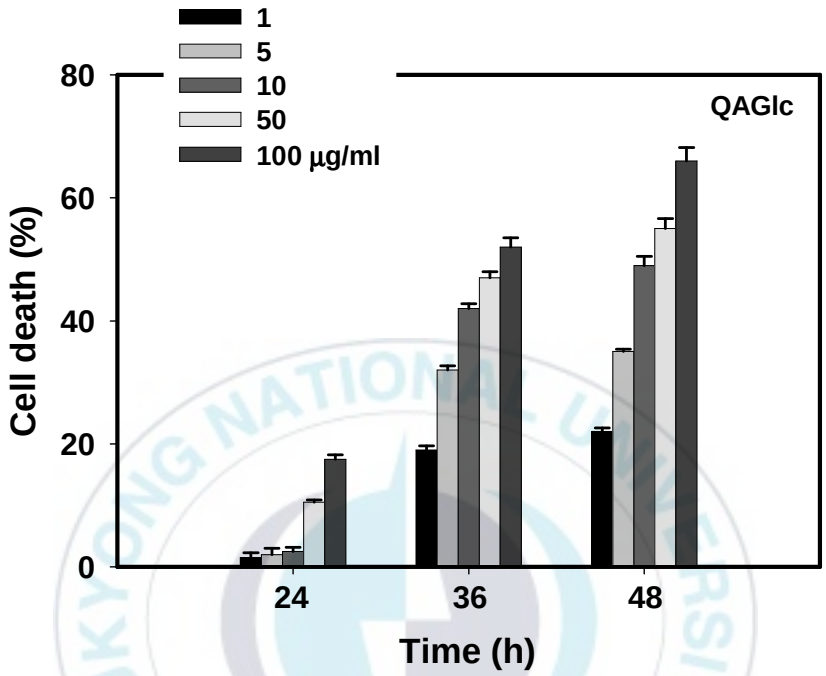


Fig. 9. Time and concentration dependant cytotoxic effects of QAGlc on B16F1 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 24 h, 36 h and 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group − OD of treatment group / OD of non-treatment group ×100).

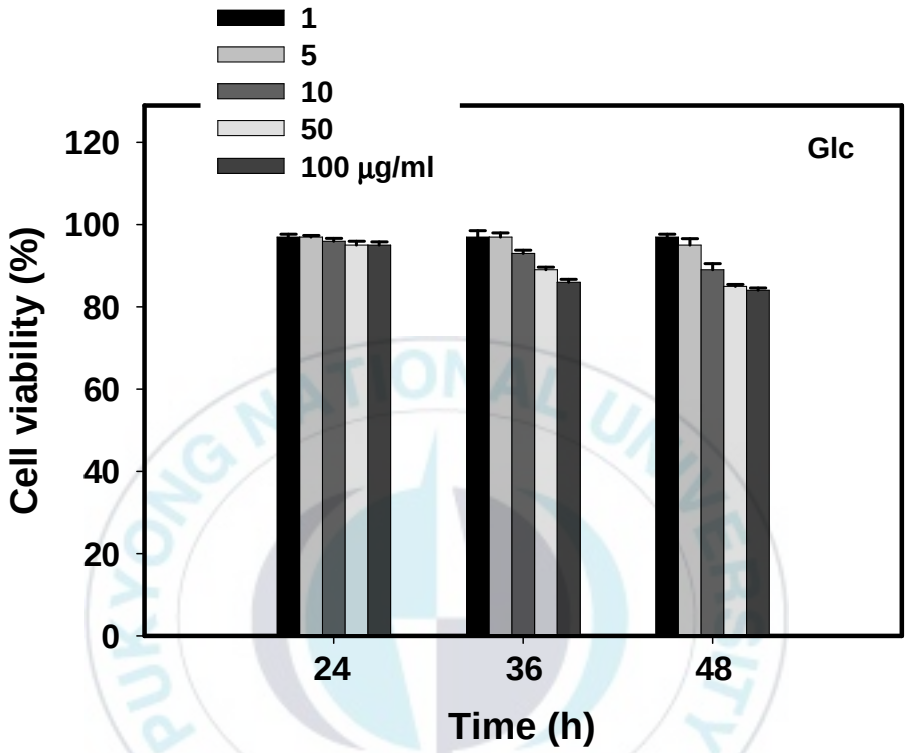


Fig. 10. Cytocompatible effect of Glc on B16F1 cells. To assess cell cytotoxicity, MTT assay was performed. Cells were grown at a density of 5×10^4 cells/well and treated with different concentrations of QAGlc. After 24 h, 36 h and 48 h of incubation, cells were added with MTT and incubated for 4 h. Finally, DMSO was added and amount of formazan salt was determined by measuring the OD at 540 nm. Viability of cells was quantified as a percentage compared to non-treatment group (OD of non-treatment group − OD of treatment group / OD of non-treatment group ×100).

4. Cytotoxic effects of QAGlc observed in B16F1 cells were due to induction of apoptosis

To ascertain whether QAGlc has a potency to induce apoptosis in B16F1 cells, DNA fragmentation assay was performed and the results are shown in Fig. 11. After 48 h of treatment a typical DNA ladder pattern due to generation of approximately 200-bp multimers by the endonucleolytic digestion was observed in treatment groups following electrophoresis. A clear DNA fragmentation effect was observed in all concentrations of QAGlc in a concentration dependent manner. These results clearly showed that QAGlc induce apoptosis in B16F1 melanoma cells. Further time dependent DNA fragmentation assay was performed in B16F1 melanoma cells with similar concentrations. A clear DNA fragmentation induction effect was observed after 36 h treatment of QAGlc confirming time dependent effect of QAGlc to induce apoptosis (Fig. 12).

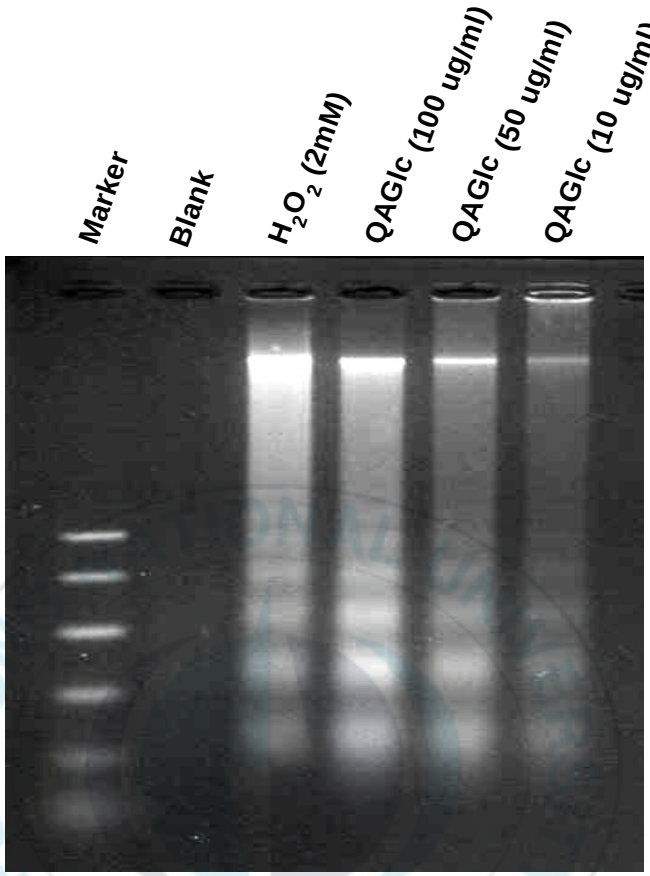


Fig. 11. DNA fragmentation assay performed in the presence of different concentrations of QAGlc. B16F1 cells (10^7 cells/ml) were treated with QAGlc at predetermined concentrations. After 48 h, fragmented DNA was extracted as a pellet using Phenol-chloroform-isoamylalcohol DNA extraction method. Fragmented DNA was analyzed by electrophoresis on a 1% agarose gel (1 h at 80 V/30 mA) in the presence of ethidium bromide using Tris-acetate running buffer.

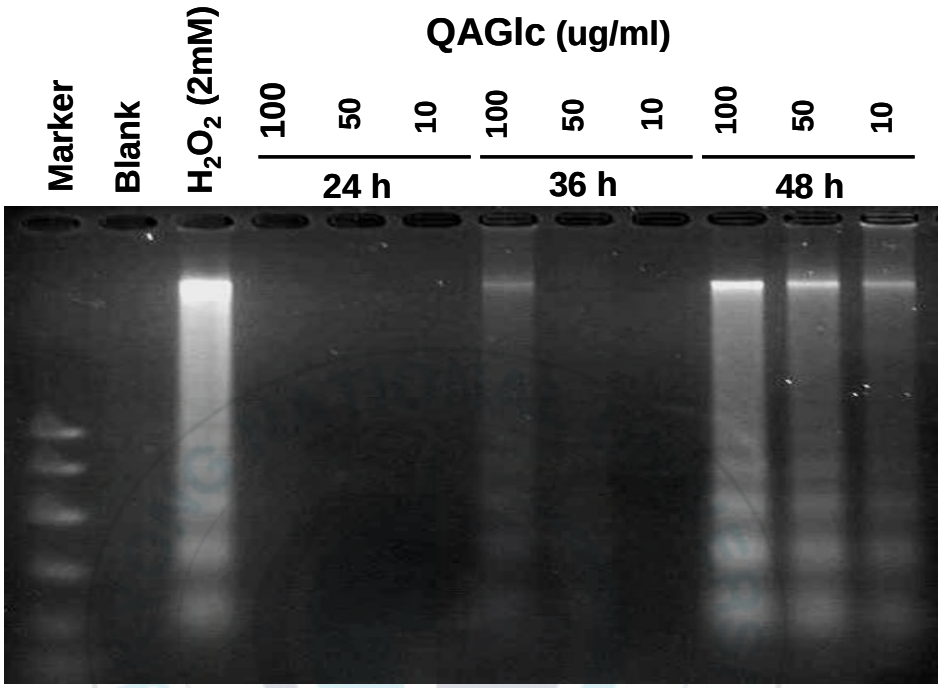


Fig. 12. Time dependant DNA fragmentation induced by QAGlc in B16F1 cells. B16F1 cells (10^7 cells/ml) were treated with QAGlc at predetermined concentrations for different time durations. After 48 h, fragmented DNA was extracted as a pellet using Phenol-chloroform-isoamylalcohol DNA extraction method. Fragmented DNA was analyzed by electrophoresis on a 1% agarose gel (1 h at 80 V/30 mA) in the presence of ethidium bromide using Tris-acetate running buffer.

5. Visualization of apoptotic cells by TUNEL assay

To further confirm the ability of QAGlc to induce apoptosis, cells were treated with QAGlc at different conditions and the results are shown in Fig. 13. Positive, pyknotic nuclei were evident in the QAGlc treated cells (Fig. 13), whereas no staining was detected in untreated cells. A clear increase in the percentage of TUNEL-positive cells was observed in a concentration dependent manner in cells treated with QAGlc for 48 h.

6. Determination of apoptosis by cell cycle analysis

To study how QAGlc affect the cell cycle progression, following treatment of QAGlc at 50 µg/ml concentration melanoma cells were monitored for 48 h. Cell cycle analysis data obtained with PI staining revealed that cell cycle progression was delayed at G1 phase after 24 h of treatment (Fig 14). However significant number of apoptotic cells was observed after 36 h of treatment and it progressed with time (Fig 15). No apoptosis was detected in untreated controlled cells. QAGlc induced apoptosis was further quantitatively analyzed by flow cytometry following staining with Annexin-V and PI. Figure 16 shows the correlation over time between the FITC-Annexin V/PI bivariate flow cytometry analysis The FITC+/PI- apoptotic cell population increased gradually after 24 h. Treatment of B16F1 cells with QAGlc for 24 h was due to the increment of early apoptotic cells. A higher number of early apoptotic cells were observed when cells were incubated for 36 h and it was greatly increased after 48 h of treatment. The total percentage of apoptotic cells (lower left + lower right quadrants) increased after QAGlc treatment in a time dependent manner, whereas only a very few cells were found in non-treated control cells. In the early stages of apoptosis changes occur at the cell surface, which have remained difficult to recognize. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Since annexin V is a Ca2+ dependent phospholipid-binding protein with high affinity for PS this protein can be used as

a sensitive probe for PS exposure upon the cell membrane (Engeland *et al.,* 1996). However, translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane looses its integrity and becomes leaky. Therefore the measurement of Annexin V binding to the cell surface as indicative for apoptosis was performed in conjunction with PI dye exclusion test to establish integrity of the cell membrane. The lower left quadrant of the cytograms shows the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for FITC-Annexin V binding and showing PI uptake. The upper left quadrant represents the apoptotic cells, FITC Annexin V positive and PI negative, demonstrating Annexin V binding and cytoplasmic membrane integrity.

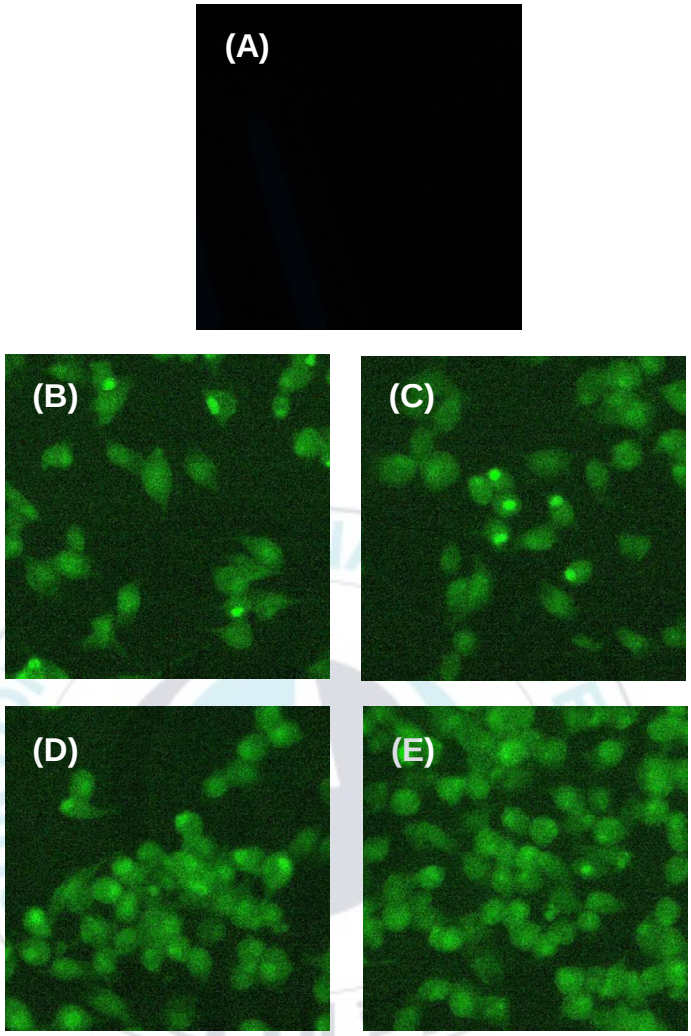


Fig. 13. TUNEL stained cells after 48 h of QAGlc treatment. (A) Blank, (B) 5 μg/ml QAGlc, (C) 10 μg/ml QAGlc, (D) 50 ug/ml and (E) 100 μg/ml. B16F1 cells (1 x 10^5) were treated with different concentrations of QAGlc and after 48 h TUNEL assay was employed to identify apoptotic cells having free 3'-OH of cleaved DNA. TUNEL assay was performed using a kit according to manufacturer's instructions described in method. FITC-labelled cells were visualized with fluorescent microscope and compared with non-treated cells.

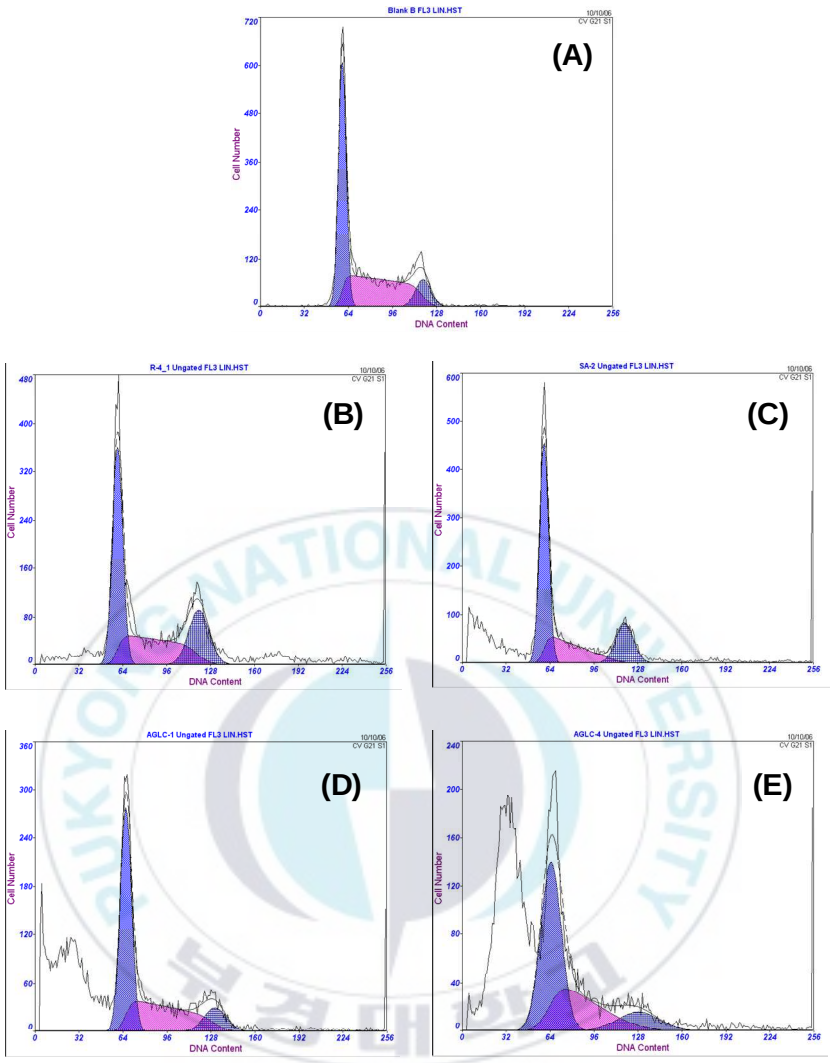


Fig. 14. Cell cycle analysis of B16F1 cells in the presence of QAGlc. Cells were treated with 50 μg/ml of QAGlc and harvested after (A) 0 h, (B) 12h, (C) 24 h, (D) 36 h, and (E) 48 h. Propidium iodide staining was used to quantify cells undergoing apoptosis and to analyze cell cycle using flow cytometer.

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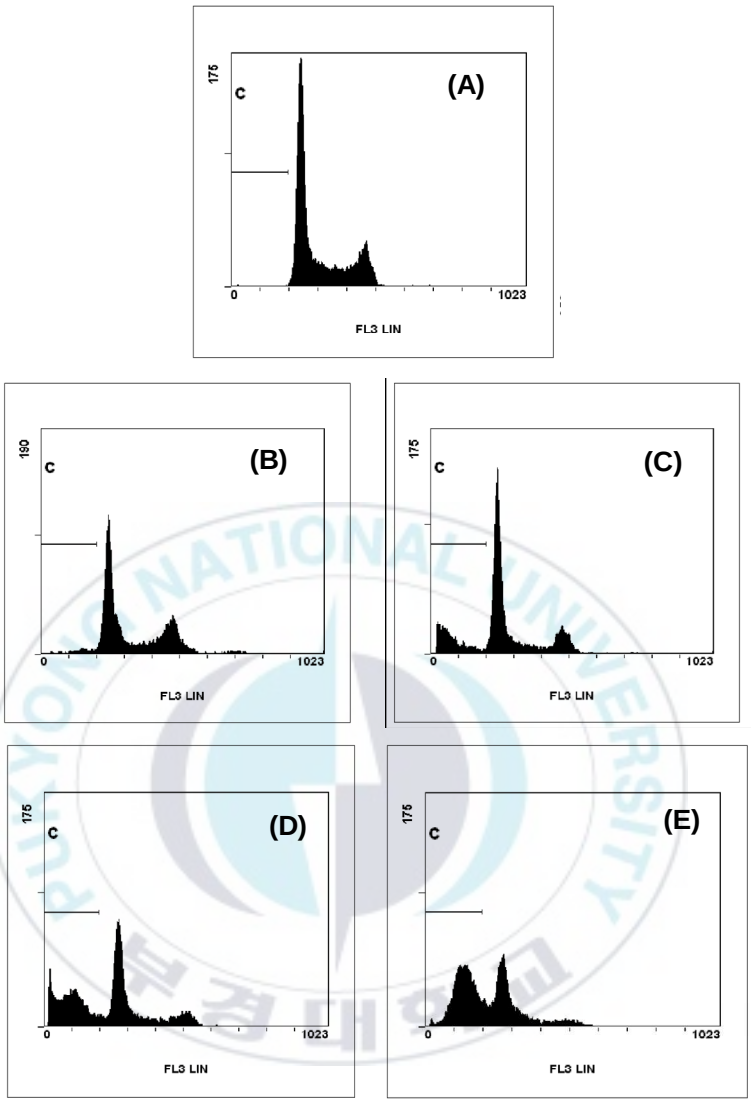

Fig. 15. Quantification of apoptosis in QAGlc treated B16F1 cells after PI staining. Cells were treated with 50 μg/ml of QAGlc and harvested after (A) 0 h, (B) 12h, (C) 24 h, (D) 36 h, and (E) 48 h. Propidium iodide staining was used to quantify cells undergoing apoptosis and to analyze the cell cycle. Fluorescence emitted from PI intercalated with DNA was measured using flow cytometer.

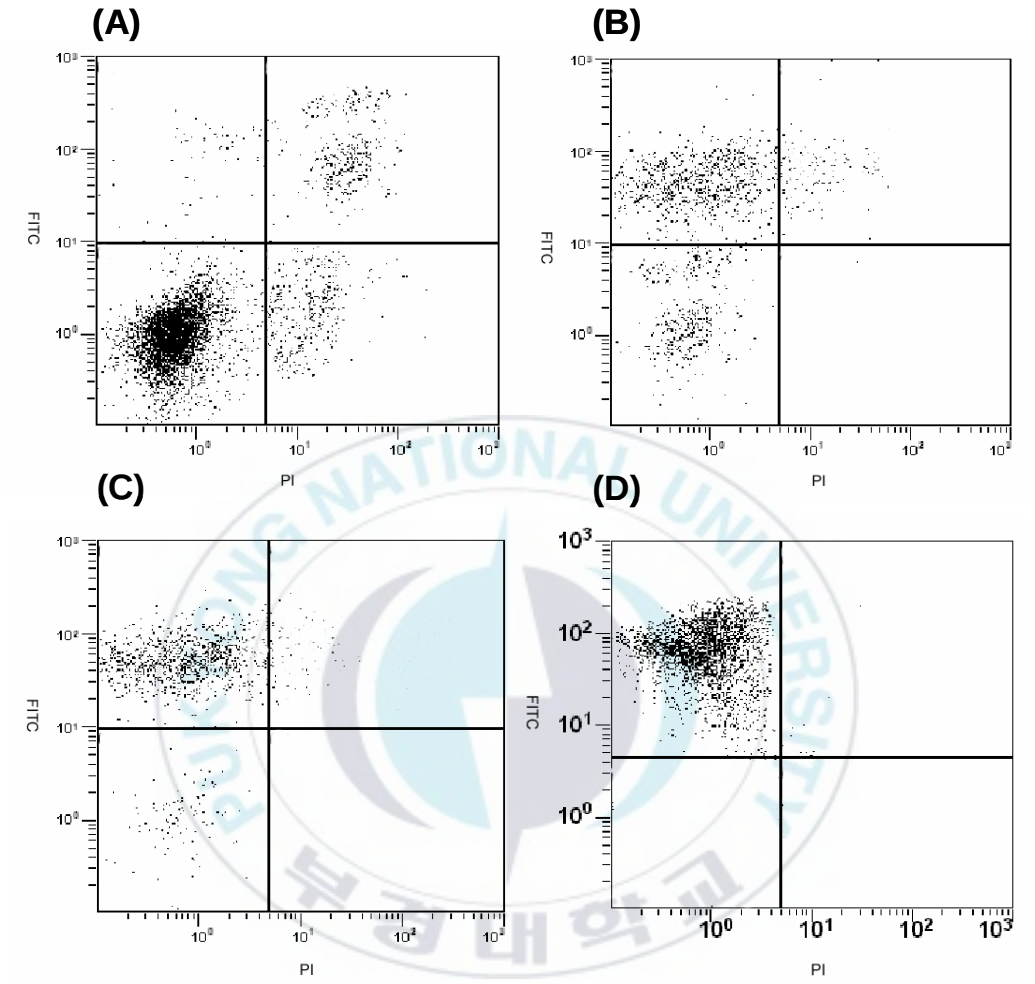

Fig. 16. Apoptosis quantification in QAGlc treated B16F1 cells following combined staining of PI and Annexin-v. B16F1 cells were treated with 50 μg/ml of QAGlc for (A) 0 h, (B) 24 h, (C) 36 h and (D) 48 h. Harvested cells were labeled with fluorescein isothiocyanate-conjugated annexin-V. After 15-min, propidium iodide was added and the percentage of viable and dead cells were determined by using the FL1 channel for annexin-V and the FL2 channel for PI using XL-MCL™ flow cytometer.

7. QAGlc induces apoptosis via induction of p53 and its target genes p21, PUMA and suppressing MDM2

To determine the possible role of p53 in induction of apoptosis by QAGlc in B16F1 cells induction of p53 was assessed at different gene expression levels. The level of p53 transcription was quantified using RT-PCR performed with primer corresponding p53 gene. Data revealed that QAGlc is a potent activator of p53 gene expression in a time dependent manner (Fig. 17). In the presence of QAGlc p53 m-RNA expression increased in a time dependent manner. Moreover, this induction was much higher than the positive control, etoposide used in this experiment. Further, reporter gene assay was carried out following treatment of QAGLc to test the induction of p53 promoter activity in p53 promoter containing luciferase vector transfected melanoma cells. The features in the map of the luciferase reporter vectors used in this experiment are shown in Fig. 18. Transfection efficiency was assessed based on the number and intensity of stained cells in the presence of X-gal. The production of blue color is due action of beta-galactosidase enzyme expressed in expression vector β-gal, on its substrate X-gal. In the present study, transient transfection was highly successful and nearly 95% transfection efficiency could be observed (Fig. 19A). The treatment of QAGlc at concentrations 100 µg/ml, 50 µg/ml, 10 µg/ml and 1 µg/ml resulted in a dose-dependent increment in the luciferase activity following 48 h of incubation with QAGlc (Fig. 19B). This luciferase activity more closely resembles the actual regulation of p53 gene promoter activity within cells because the putative cis-acting enhancer elements are existed in cloned promoter region of the vector.

Since the mechanism of p53 activation in response to apoptosis inducing agents involves mainly posttranslational modifications, including phosphorylation and acetylation (Giaccia *et al.,* 1998), western blot analysis was conducted to analyze the induction of total p53, phospho p53 and acetylated p53 using respective antibodies following treatment of QAGlc for different time intervals. Anticancer effects of etoposide are reported to be high after 24 h treatment (Karpinich *et al.,* 2002). Therefore in this experiment to compare the effects

etoposide was treated for 24 h. Protein expression of all three forms of p53 was increased in a time dependent manner (Fig. 20). However, among three forms, significantly increased time dependent induction was observed in acetylated p53 levels. Moreover, phosphor-p53 level was also increased significantly in a time dependant manner. Induction of both these active forms of p53 was much higher than protein expression levels in the presence of etoposide after 24 h. These data clearly indicated the stabilization in protein expression levels of activated p53 forms. Several studies have suggested that acetylation sites play a critical role in Mdm2- mediated ubiquitination and subsequent degradation of p53 (Rodriguez *et al.*, 2000). The observation that QAGlc stabilizes the active forms of p53 and subsequently induce apoptosis are in agreement with the reported results that , increasing levels of p53 acetylation with deacetylase inhibitors found to prevent p53 from degradation in vivo (Li *et al.*, 2002), indicating that acetylation of p53 may directly regulates its stability to induce apoptosis.

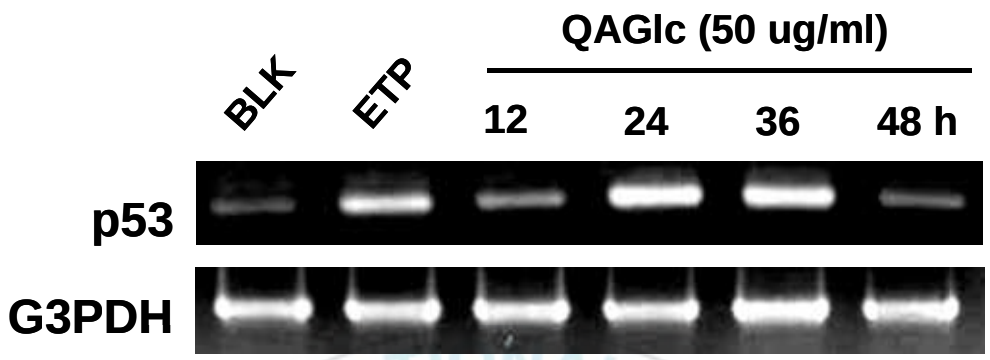

Fig. 17. p53 mRNA expression in QAGlc treated B16F1 cells. QAGlc treated B16F1 cells were incubated for different time durations and total RNA was extracted as described in methods section. For RT-PCR, 1 μg of total RNA was reverse-transcribed to generate first strand cDNA using AMV reverse transcriptase by reverse transcription (RT). RT-generated cDNA was used as a template to amplify p53 and G3PDH mRNA. Specific upstream and downstream primers were employed for the reaction. The amplified DNA fragments were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The mRNA of G3PDH served as an internal control for sample loading and mRNA integrity. BLK: non-treatment group; ETP: Etoposide.

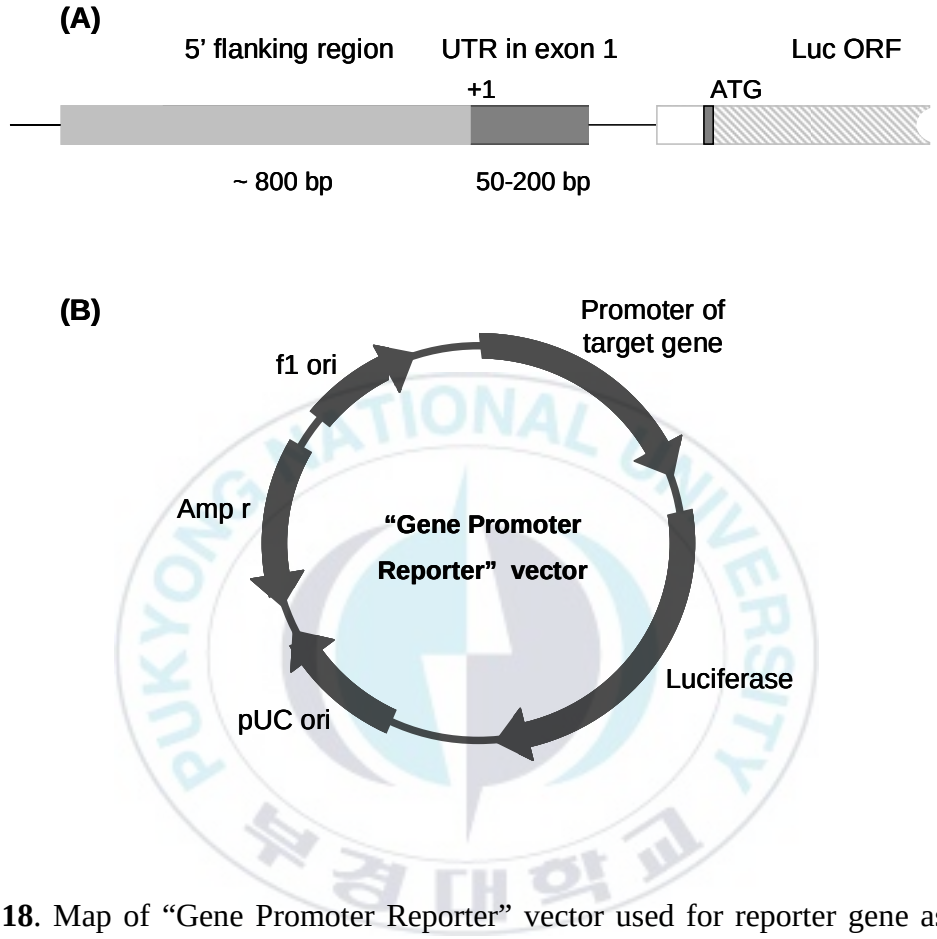

Fig. 18. Map of "Gene Promoter Reporter" vector used for reporter gene assay. (A) "Gene Promoter Reporter" vector contains approximately 800 bp of 5' flanking region and 50-200 bp of untranslated region (UTR) of exon 1 of the NF-κB gene. This ~ 1kb region is located upstream of the start codone of luciferase gene.

(B) The vector backbone contains an ampicillin-resistant (Amp r) gene which allows ampicillin selection in E. coli, a pUC origin of replication for propagation in E. coli, and f1 origin for single-stranded DNA production.

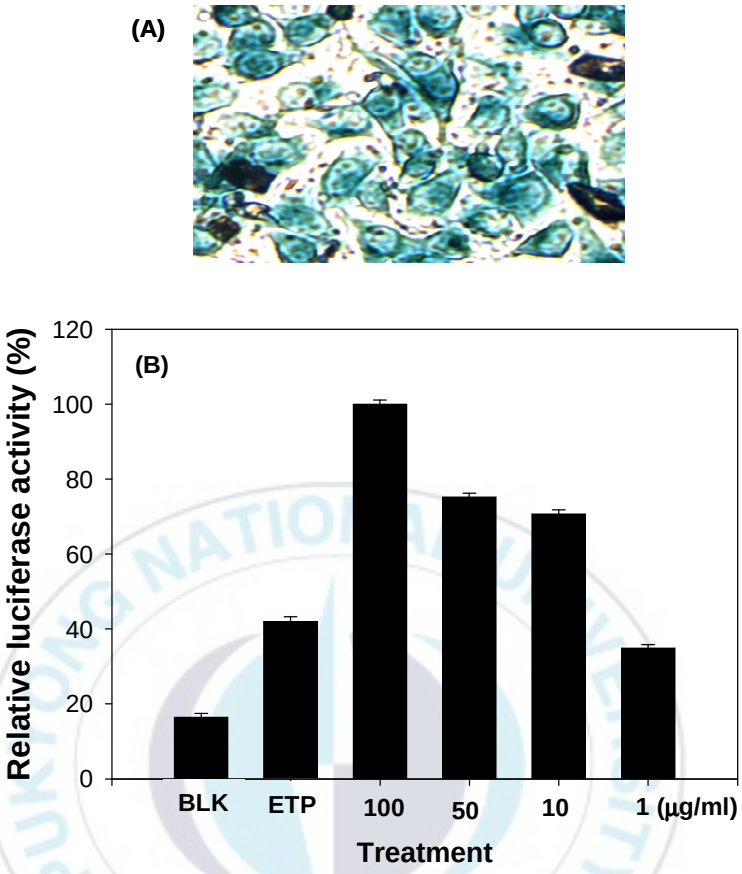

Fig. 19. Induction of p53 promoter activity in transfected cells. B16F1 cells were co-transfected with p53-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured and were treated with different concentrations of QAGlc. Luciferase enzyme activity that represents p53 promoter activity was determined using luciferin as the substrate. X-gal staining method was employed to quantify transfected cells. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity. A: Beta-galactosidase staining of transfected cells. B: Relative luciferase activity. BLK: non-treatment group; ETP: Etoposide.

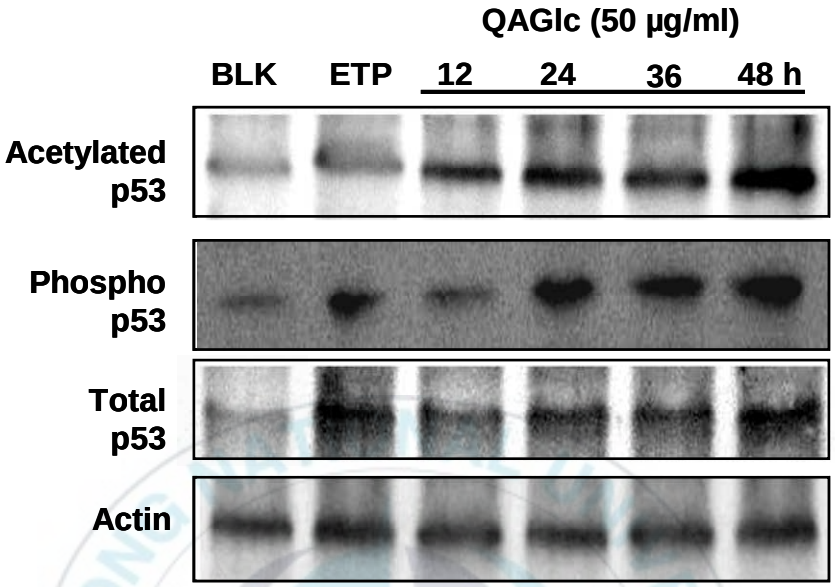

Fig. 20. Western blot analysis of p53 protein expressions in B16F1 cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; ETP: Etoposide.

8. Other target genes in apoptosis pathway that affected by the treatment of QAGlc

Since the tumor suppressor p53 inhibits cell growth through activation of cell cycle arrest and apoptosis via induction of cascade of p53 direct targets, protein expression levels of a number of genes that are critically involved in either cell growth arrest or apoptosis was assessed in the presence of QAGlc. These include p21, Mdm2, PUMA, cdks and cyclins. Western blot analysis carried out with anti MDM2 antibody exhibited that treatment of QAGlc slightly suppressed the protein expression of MDM2 (Fig. 21). This suppression is favourable for the transcriptional activation of p53 because MDM2 bind to the p53 tumor suppressor protein and inhibits its ability to act as a transcription factor (Momand *et al.*, 1992). However, contradictory results can be found in studies that have dealt with MDM2 expression. Some researchers suggest that if a chemotherapeutic agent induces p53, it causes simultaneous induction of expression of MDM2. This is because p53 also activates MDM2 expression at the level of transcription (Wu *et al.*, 1993; Barak *et al.*, 1993), suggesting that MDM2 can function as a negative feedback regulator of p53. However, in this research, even though QAGlc is not capable of suppressing MDM2 to a greater extent its ability to greatly enhance the stability of p53 through phosphorylation and acetylation promote its p53 dependent apoptosis function.

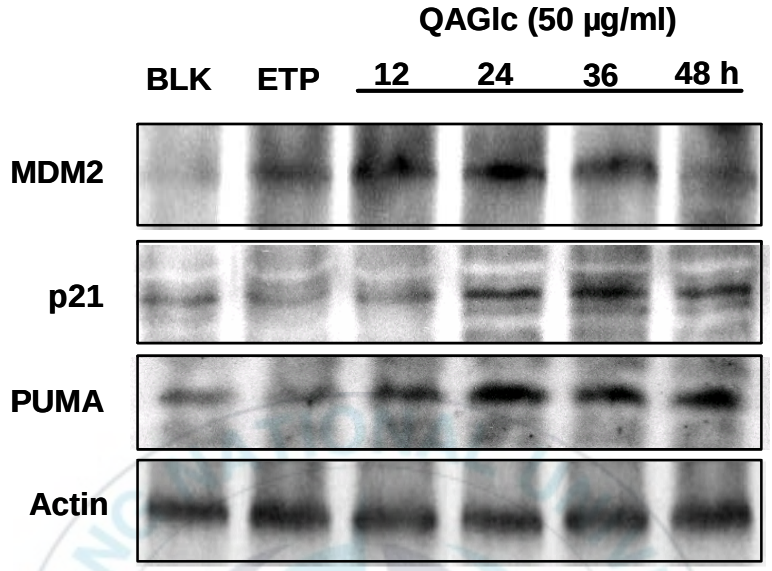

Fig. 21. Western blot analysis of MDM2, p21 and PUMA protein expressions in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; ETP: Etoposide.

When the effect of QAGlc on the induction of p21 was assessed, the protein expression level of p21 was increased in a time-dependent manner in the presence of QAGlc (Fig. 21). However, induction of p21 could not be observed with the treatment of etoposide. These data are in agreement with the reported data that expression of p53 induces growth arrest via transcriptional activation of the cyclin-dependent kinase inhibitor p21. Further, effect of the treatment of QAGlc on the promoter activity of p21-promoter containing luciferase vector transfected B16F1 cells were assessed using reporter gene assay. A dose-dependent induction of luciferase activity that represents p21 promoter activity in transfected cells was observed when cells were treated with QAGLc. Up to 4-fold increment was observed at the optimal concentration of 100 μg/ml concentration (Fig. 22). Accordingly, it was speculated that growth inhibitory effect of QAGlc is at least partly mediated by QAGlc induced p21 pathway by arresting progression of cell cycle arrest at G_1 phase.

Western blot analysis performed with anti-PUMA antibody clearly exhibited that QAGlc induced PUMA protein expression (Fig. 21). This was expected because PUMA has an expression pattern consistent with a causative role in p53-dependent apoptosis (Yu *et al.*, 2001). The protein encoded by PUMA was exclusively localized to mitochondria where it interacted with Bcl-2 which is a potent cell death-suppressor (Reed *et al.*, 1994) and represents a unique type of protooncogene that extends cell survival by inhibiting apoptosis rather than promoting cell proliferation (Korsmeyer *et al.*, 1992). Treatment of QAGlc resulted time dependent suppression of Bcl-2 protein expression (Fig. 23). In contrast, Bax was up-regulated in a time dependent manner (Fig. 24). However, after 36 h of treatment substantial increment in Bax expression compared to untreated group was observed. These data suggested that QAGlc induced apoptosis partly by the alteration of the ratio of BCl-2/Bax family protein expression that directly affects release of cytochrome C via alteration of mitochondrial membrane permeability.

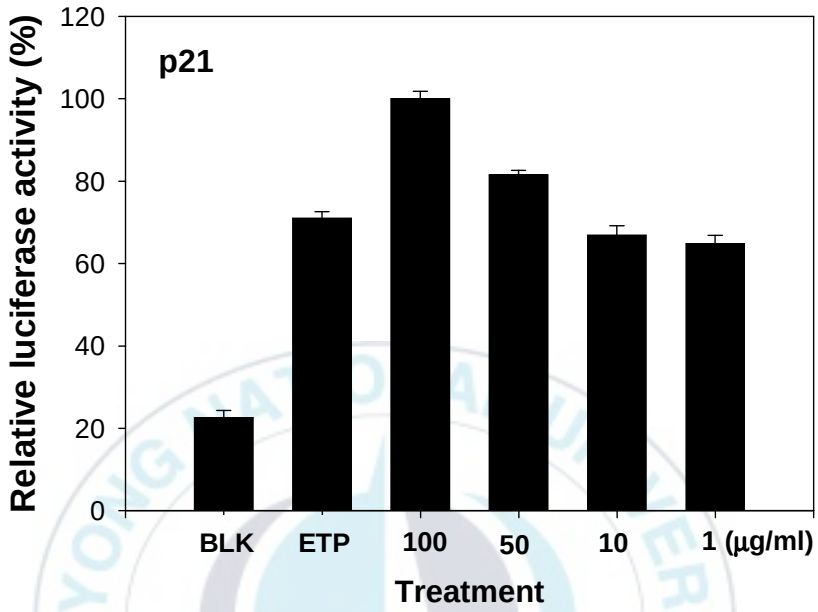

Fig. 22. Induction of p21 promoter activity in transfected cells. B16F1 cells were co-transfected with p21-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured and treated with different concentrations of QAGlc. Luciferase enzyme activity that represents p21 promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity. BLK: non-treatment group; ETP: Etoposide.

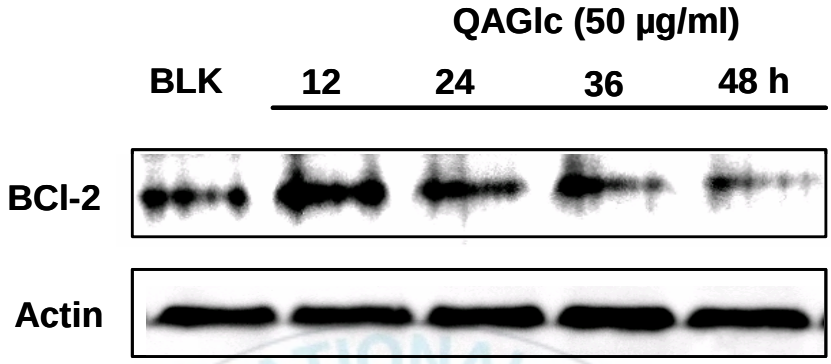

Fig. 23. Western blot analysis of BCl-2 protein expression in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group.

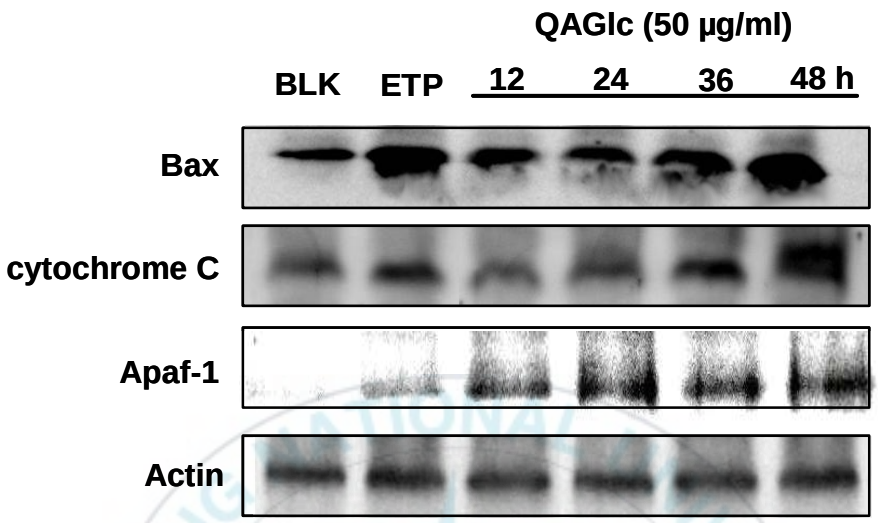

Fig. 24. Western blot analysis of Bax, cytochrome C and Apaf-1 protein expressions in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; ETP: Etoposide.

Further, QAGlc clearly induced the cytochrome C and Apaf-1 protein levels in B16F1 cells in a time dependent manner (Fig. 24). Also this induction followed a similar pattern to the induction of Bax. A clear induction of Bax as well as cytochrome C can be seen after 36 h. These data are in agreement with the fact that during apoptosis, cytochrome C is released from mitochondria into the cytoplasm, by the Bax via alteration of membrane permeability (Li *et al.,* 1997). Moreover, Bax and cytochrome C protein levels after 48 h of QAGlc treatment were much similar to protein levels in the presence of etoposide. With this observation, in order to assess the effect of QAGlc to induce Apaf-1, protein expression levels of Apaf-1 in the presence of QAGlc were tested following the same treatment durations. A significant induction of Apaf-1 protein compared to that of non-treated group was observed following treatment of QAGlc to cells. In untreated group a slight expression level was observed whereas QAGlc increased the expression of Apaf-1 protein level in a time dependent manner. The central importance of this study is manifested by the observation that Apaf-1 is induced following treatment of QAGlc becasue in cancers such as malignant melanoma (Soengas *et al*., 2001) it is frequently mutated.

Further, concurrent induction of caspase-9 expression was observed in the presence of QAGlc (Fig. 25). This was an expected result because processed caspase-9 remains associated with the apoptosome as a holo-enzyme to maintain its catalytic activity, (Rodriguez *et al.,* 1999) and the apoptosome serves as an allosteric regulator for the enzymatic activity of caspase-9. Induction of members in the apoptosome in the presence of QAGlc confirmed its apoptotic and anticancer potential since Apaf-1/caspase-9 pathway mediates a variety of apoptotic stimuli including those initiated by the activation of tumor suppressor proteins and oncogenes.

In line with the induction of capase 9 a simultaneous induction of caspase-3 protein level was observed in western blot data proceeded with anti-caspase-3 antibody (Fig. 25). Apoptosis is executed by a cascade of caspase activation (Thornberry *et al.,* 1998). The activation of an effector caspase, such as caspase-3,

is performed by an initiator caspase, such as caspase-9, through proteolytic cleavage at specific Asp residues. Therefore activation of initiator caspase, caspase-9 in the presence of QAGlc can activate the effector caspases and subsequently cleave numerous cellular targets, leading to cell death. Moreover, in order to confirm the effect of QAGlc on the function and activation of caspase members functioning in extrinsic pathway of apoptosis, protein level of caspase-8 was assessed following treatment of QAGlc in a time dependent manner. However, the caspase-8 level was not changed with the treatment of QAGlc confirming QAGlc cannot exert any effect via death receptor signaling pathway (Fig. 25).

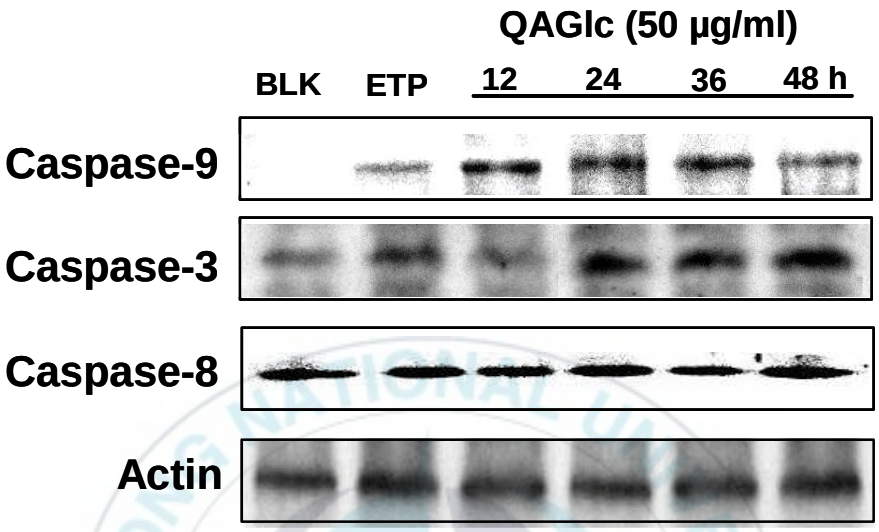

Fig. 25. Western blot analysis of caspase-9, caspase-3 and caspase-8 protein expressions in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; ETP: Etoposide.

9. QAGlc exerted inhibitory effects on proteins that are necessary for the progression of the cell cycle

In order to confirm the potency of QAGlc to arrest progression of cell cycle, cyclin D, E and cdk2/4/6 levels were assessed. It was worth assessing these protein levels in the presence of QAGlc, because protein expressions of the cyclins and cdks, are regulated by p21. QAGlc treatment of the cells resulted in a dose- and timedependent decrease in protein expressions of cyclin D and cyclin E (Fig. 26) as well as cdk2, cdk4, and cdk6 (Fig. Fig. 25). In the time-dependent study, the decrease in cyclin D protein was more pronounced than that of cyclin E. In the time-dependent study, however, the levels of cyclin D was found to be inhibited by QAGlc treatment as early as 12 h after treatment, but the inhibitory effect of the same treatment on cyclin E was evident only after 24 h of treatment. Similarly, the decrease in the protein expression of cdk4 and cdk6 was found to be more pronounced than that of cdk2 (Fig. 27).

Since the retinoblastoma protein (Rb) is the major target of cyclinD1-cdk4/6 for cell cycle regulation and is also present in mutant form in a number of cancers (Sherr *et al.,* 1994) its protein expression level was studied in the presence of QAGlc. Interestingly and as expected, treatment of QAGlc into B16F1 cells induced RB expression (Fig. 28). Since the levels of cdk2, 4 and 6 levels that are were decreased in the presence of QAGlc it would lead the phosphorylation of RB and RB-mediated passage through the G_I phase of the cell cycle. The retinoblastoma (RB) family of proteins has been shown to regulate the activity of members of the E2F family of heterodimeric transcription factors in a variety of cell culture studies. In agrrement with the fact that RB proteins bind directly to E2F proteins and repress E2F-mediated transcription, induction of Rb protein expression followed a suppressed E2F protein expression (Fig. 28). This further confirms the cell cycle arrest in the presence of QAGlc because regulation of the various E2F family proteins potentially links transcriptional activation and repression to the control of cell cycle progression. The activity of the E2F proteins is known to be regulated in a cell cycle-dependent manner, and fluctuations in

E2F activity enable the coupling of an intricate web of gene expression programs with cell cycle position (Dyson, 1998). Therefore taken together these results confirm the ability of QAGlc to arrest cell cycle progression at the G1-S boundry. Moreover effects of QAGlc on cell cycle related protein expression levels are in line with similar potency bearing anticancer compounds.

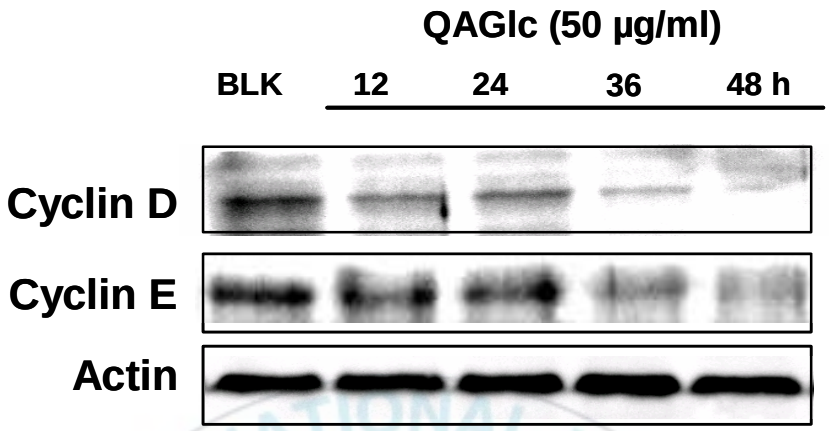

Fig. 26. Western blot analysis of cyclin D and cyclin E protein expressions in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group.

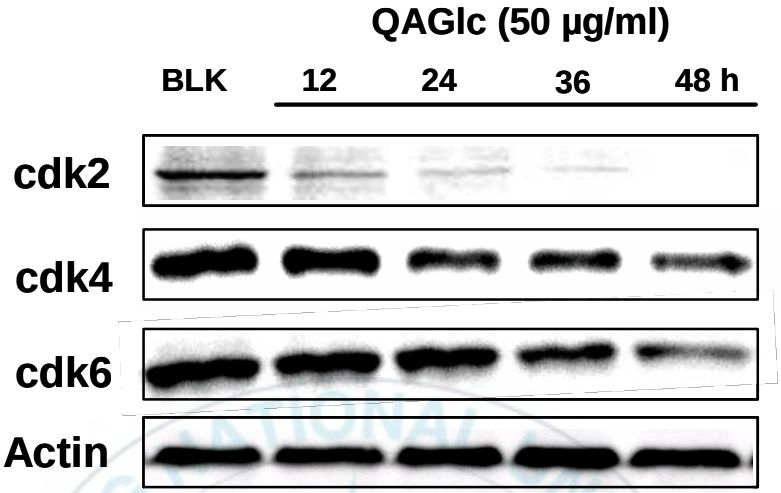

Fig. 27. Western blot analysis of cdk2, cdk4 and cdk6 protein expressions in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group.

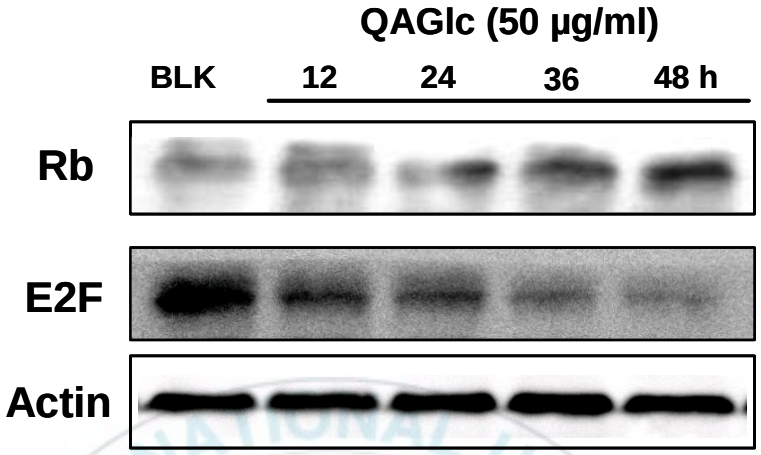

Fig. 28. Western blot analysis of Rb and E2F protein expression in B16F1cells treated with QAGlc. After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group.

10. The proposed special feature of QAGlc mediated apoptosis in B16F1 melanoma cells

During this study it was observed that QAGlc exerted a higher cytotoxic effect on B16F1 cells but the positive control etoposide could not kill B16F1 cells by inducing apoptosis. This observation is in agreement with reported findings that inactivation of apaf-1 in melanoma promotes its chemoresistance in the presence of well known anticancer agents. Indeed, cells within melanoma lesions reported to possess an inherently low level of spontaneous apoptosis, and resistance to apoptosis that correlated with increased metastatic potential in animal models of melanoma (Fujimoto *et al.*, 2004). Therefore a few number of anticancer drug agents have become effective in melanoma cell progression. There is also evidence for increased resistance to apoptosis in melanocytic nevi compared to isolated melanocytes, suggesting that the acquisition of apoptosis resistance may be an early step in the malignant transformation from normal melanocyte to melanoma (Soengas *et al.*, 2001). However, treatment of etoposide to B16F1 cells led to clear induction of functional p53 levels. This led a necessity to elucidate the target protein that essentially altered only in the presence of QAGlc, but not in the presence of etoposide that favors QAGlc mediated apoptosis in melanoma. Careful assessment of apoptosis related protein expression levels in the presence of QAGlc and etoposide suggested that etoposide was not effective to activate Apaf-1 protein expression that was greatly induced in the presence of QAGlc. These results suggested the notion that QAGlc is positively involved in induction of apoptotic related events mainly via induction of Apaf-1.

Therefore to obtain detailed mechanistic evaluation, B16F1 cells and other cancer cells used in this study were treated with etoposide. Following incubation for 48 h protein levels of Apaf-1 and caspase-9 were assessed using western blot analysis. As expected a clear decrement in expression level of Apaf-1 was observed in B16F1 cells after 48 h of etoposide treatment (Fig. 29). All the other cell lines exhibited a clear expression of Apaf-1 in the presence of etoposide. Simultaneously, all other cell lines except B16F1 cells expressed higher levels of

caspase-9 protein expression proving better functionaing of apatosome. This partly cleared the mechanism of QAGlc in B16F1 cells that it induces apoptosis in this cell line via induction of Apaf-1. Because, Apaf-1 has been shown to be a key element involved in the mitochondria-dependent apoptosis, that bind to cytochrome C and favoring activation of caspase-9. Further, the study was elaborated with the treatment of QAGlc and etoposide separately. QAGlc and etoposide were treated simultaneously in a separate other experiment to B16F1 cells in a time-dependent maneer. However, etoposide could induce neither Apaf-1 nor caspase-9 in B16F1 cells when it was treated to cells alone (Fig. 30). Further, the results demonstrated that QAGlc induced Apaf-1 and caspase-9 levels in a time-dependent manner. However, simultaneous treatment of QAGlc and etoposide clearly increased both Apaf-1 and caspase-9 protein levels indicating the ability of QAGlc to induce apoptotic potency in B16F1 cells via activation of Apaf-1. Taken together, overall effects of QAGlc in B16F1 cells that led apoptosis are illustrated in a proposed apoptotic signaling cascade (Fig. 31).

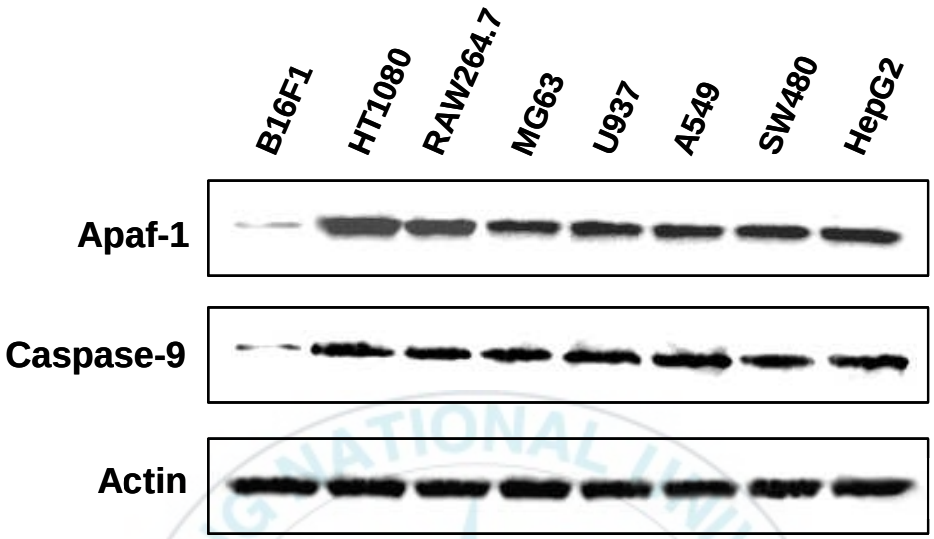

Fig. 29. Western blot analysis of Apaf-1 and caspase-9 protein expressions in different cancer cell types treated with etoposide. After treatment 48 h, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein.

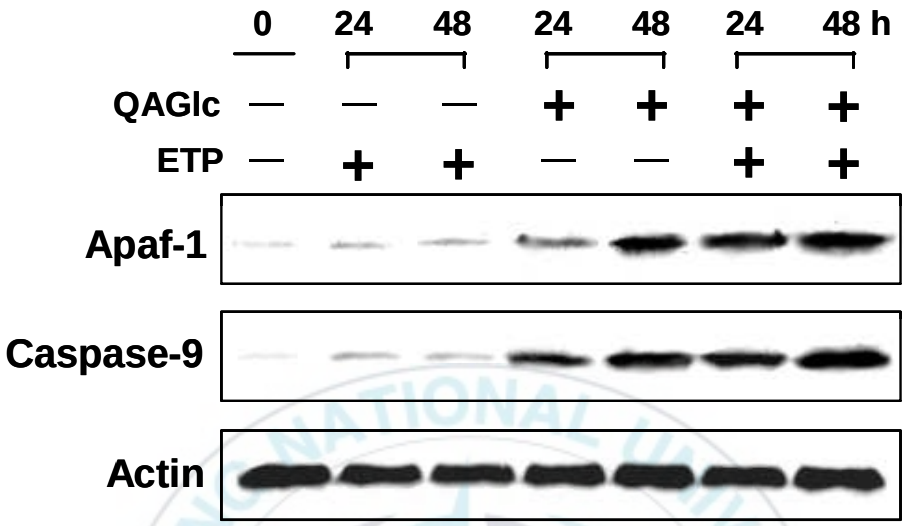

Fig. 30. Western blot analysis of Apaf-1 and caspase-9 protein expressions in B16F1cells treated with etoposide and QAGlc in a predetermined pattern. After treatment, for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. ETP: Etoposide.

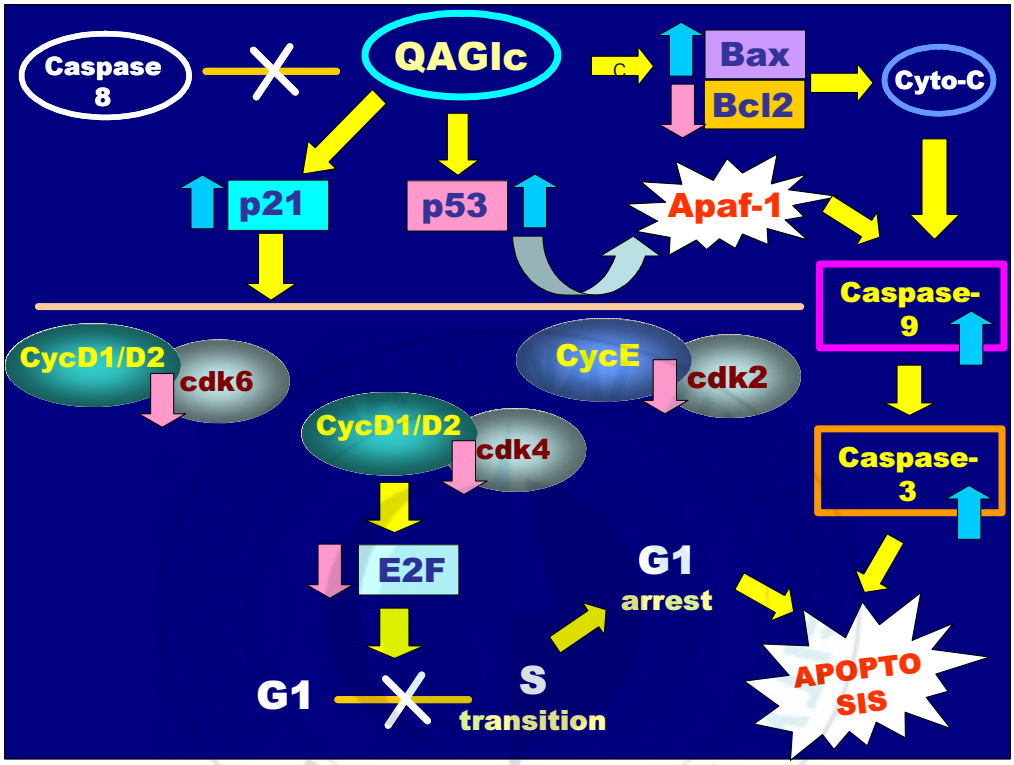

Fig. 31. The proposed signaling cascade of the effects of QAGlc in B16F1 cells.

11. Summary

In the present research glucosamine was used as the starting material to synthesize a novel cationic glucosamine derivative having quaternized amino functionality. This novel glucosamine derivative exerted a cell specific cytotoxic effect in B16F1, murine skin cancer cell line in a time and concentration dependent manner. DNA fragmentation analysis confirmed that QAGlc kills B16F1 cells by inducing apoptosis. Further, cell cycle analysis of B16F1 cells revealed that cell cycle progression was delayed at G_1 phase in the presence of QAGlc. Moreover, apoptosis quantification studies presented the increment in subcellular G_0 phase that represent cells undergoing apoptosis after treatment with QAGlc. Western blot analysis performed to study protein expression levels exhibited that active forms of p53 gradually increase with time when the cells were treated with QAGlc leading to the stabilization of p53 function in melanoma cells. Further, reporter gene assay results confirmed the ability of QAGlc to induce promoter activity of p53 in melanoma cells. Concurrently, induction in protein expression levels of cytochrome C, Apaf-1 and caspase-9 confirmed QAGlc followed a mitochondrial pathway of apoptosis in B16F1 cells. This was facilitated with the alteration of mitochondrial membrane permeability that determined from the induction of Bax and decrement in anti-apoptotic protein Bcl-2. Time-dependent increment in cdk inhibitor, p21, led subsequent reductions in E2F, cyclin D/E and cdk2/4/6 levels disturbing transition of cell cycle from G_1 to S phase. However, unchanged protein expression level of caspase-8 revealed that QAGlc could not act to induce apoptosis by leading death receptor signaling pathway. Further, induction in Apaf-1 level in the presence of QAGlc found to be affecting greatly to induce cascade of signaling events subsequently resulting apoptosis. Therefore, this study gives an insight into the signaling pathway and a possible mechanism of positively charged small molecule that exert anticancer effects including apoptosis.

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Part 2

MMP-2 and MMP-9 Inhibition by Quaternized Amino Glucosamine in HT1080, Fibrosarcoma Cells

Introduction

During the last few decades, the discovery of new anticancer agents has been targeted based on the development of compounds that interfere with nonspecific intracellular processes. More recently, new understanding of tumor cell biology has permitted the identification of such cellular processes that are specifically altered in cancer cells and responsible for autonomous growth and replication, invasion of surrounding tissues, and formation of metastases. These abnormal processes, some of which can be targeted therapeutically, include the over-expression of molecules involved in tumor invasion and metastasis. Drugs targeted to these processes are expected to specifically disrupt the growth of malignant tumors without altering normal tissues and, therefore, to cause minimal toxicity. Many such agents are currently undergoing clinical evaluation. Potential targets for new drugs include the matrix metalloproteinases (MMPs), a group of proteinases that have physiologic roles in degrading and remodeling the extracellular membrane.

1. MMPs: Definition, function and regulation

The MMPs are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all of the components of the extracellular matrix (Chambers *et al.*, 1997). The MMPs were originally described as the enzymes responsible for dissolution of the tadpole tail, and subsequent studies have indicated that these proteases, which are synthesized by connective tissue cells, are important for the remodeling of the extracellular matrix that accompanies physiologic processes, such as uterine involution, bone resorption, and wound healing (Kahari *et al.*, 1999; Ray *et al.*, 1995; Kleiner *et al.*, 1999). The human MMP gene family consists of at least 18 structurally related members that fall into five classes according to their primary structure and substrate specificity: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12),

membrane type (MT)-MMPs (MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP), and nonclassified MMPs (Kleiner *et al.*, 1999). The general structure of the MMPs includes a signal peptide, a propeptide domain, a catalytic domain with a highly conserved zinc-binding site, and a haemopexin-like domain that is linked to the catalytic domain by a hinge region. In addition, MMP-2 and MMP-9 contain fibronectin type II inserts within the catalytic domain, and MT-MMPs contain a transmembrane domain at the C-terminal end of the haemopexin-like domain. The haemopexin domain is absent in the smallest MMP, matrilysin (MMP-7). MMPs are highly regulated at the levels of both gene expression and protein activation. Transcriptional regulation of MMP genes is mediated by an AP-1 regulatory element in their proximal promoter regions (Fini *et al.*, 1998). Most MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space. The pro-MMPs are retained in their inactive form by an interaction between a cysteine residue located in the propeptide portion of the molecule with the catalytic zinc atom, blocking the access of substrates to the catalytic pocket of the enzyme. Partial proteolytic cleavage of the propeptide dissociates the covalent bond between the cysteine residue and the catalytic site and exposes the catalytic site to the substrate. MMPs are activated in an orderly fashion, with one activated MMP cleaving and activating the next in a complex and only partially deciphered network of proteases in the extracellular space (Murphy *et al.*, 1999).

The proteolytic activity of MMPs is inhibited by the specific tissue inhibitors of the metalloproteinases (TIMPs). The TIMPs are a family of four structurally related proteins (TIMP-1, -2, -3, and -4), which exert a dual control on the MMPs by inhibiting both the active form of the MMPs and their activation process. The TIMPs inhibit the enzymatic activity of all members of the MMP family by forming noncovalent stoichiometric complexes with the active zinc-binding site of the MMPs. In addition, the TIMPs inhibit the catalytic activation of many pro-MMPs, with various members of the TIMP family having preferential inhibitory capabilities against the different pro-MMPs. For example, TIMP-1 forms

preferential complexes with pro-MMP-9, whereas TIMP-2 and TIMP-4 exhibit higher affinity for pro-MMP-2 (Gomoez *et al.,* 1997; Henriet *et al.,* 1999; Nemeth *et al.,* 1996).

2. Role of MMPs in tumor growth, invasion and metastasis

MMPs are overexpressed in multiple tumor types when compared to normal tissues (Mannello *et al*., 2005, Egeblad and Werb, 2002). The up-regulation of the MMPs may be a secondary effect of the remodeling of the matrix and/or growth characteristics of the tumor (Sternlicht and Werb, 2001). However, in cases in which increased MMP levels have been shown to be strong indicators of a negative prognosis, it is more likely that targeting those enzymes will impact tumor progression; several studies pointed to the possible use of MMPs in the future to augment treatment strategies in specific cancers (Agnantis *et al*., 2004, Mannello *et al*., 2005, Wilson *et al.,* 1997, Itoh *et al.,* 1998; Masson *et al.,* 1998). MMPs were classically thought to contribute to tumor metastasis via their matrix degrading activity, but in recent years, studies have implicated MMPs at virtually all stages of tumor progression from initial development of the tumor, growth, angiogenesis, invasion, and metastasis and growth at the secondary site (Egeblad and Werb, 2002). There are several ways in which MMPs can increase tumor cell proliferation: first of all, MMPs can release the cell-membrane-bound precursors of some growth factors; they can render bioavailability of peptide growth factors that are sequestered by ECM proteins and moreover they can control proliferation signals through integrins (McCawley and Matrisian, 2001; Martin *et al.,* 1999; Manes *et al.,* 1997). MMPs also confer anti-apoptotic characteristics to cancer cells as can be seen by the release of FasL (Mannello *et al*., 2005). For tumor cells to continue growing and to migrate to distant sites, the formation of new blood vessels is a fundamental step and many studies with endogenous and synthetic inhibitors indicate the central role MMPs occupy in this process (Hanahan and Folkman, 1996). MMPs can favor new blood vessel sprouting by simply eliminating physical barriers through the degradation of ECM structural

components, or by the generation of pro-angiogenic factors. In fact, it has been demonstrated that the cleavage of collagen type IV exposes a cryptic binding site essential for endothelial cell migration increasing the bioavailability of the pro angiogenic vascular endothelial growth factor (Xu *et al*., 2001). For tumor-cells to migrate and metastasize, it is necessary to break down several ECM barriers, and cell movement is strongly associated with proteolysis and requires bi-directional interactions between cell and ECM (Sternlicht and Werb, 2001). MMPs take part in all events that lead to tumor cell detachment, invasion of the basement membrane and surrounding stroma and colonization of new sites. Cancer cells must first detach from the primary tumor cleaving cell-surface E-cadherin and down regulating cell-cell adhesion mechanisms (Egeblad and Werb, 2002). Moreover, tumor cells must detach from the ECM and neighboring cells and the MMP-dependent cleavage of cell-matrix adhesion receptors can favor this process (McCawley and Matrisian, 2001, Xu *et al*., 2001). It is well known that cancerous cells have the capacity of resisting and escaping from immune surveillance and it is becoming evident that MMPs are involved in these evasion mechanisms (Sheu *et al*., 2001). The establishment of tumor cells in new distant sites necessitates a strong interaction between the malignant cells and the host tissue stroma. Even though tumor cells express their own pattern/set of MMPs, it is becoming evident that they can direct MMP expression by endothelial cells, fibroblasts, and also leukocytes (Stamenkovic, 2003). The MMPs secreted by the stroma are important in tumor-directed tissue remodeling, not only through physical structural modifications, but also and more importantly, through the release and increased bioavailability of molecules that can enhance tumor growth, angiogenesis and tumor cell migration (Egeblad and Werb, 2002). The mechanisms of MMP expression are very complex. MMP transcription can be regulated by growth factors, cytokines, and oncogene products, which can be released by the stroma or by tumor cells themselves (Mannello *et al*., 2005). MMPs are overexpressed in many types of cancerous tissue and often it is not the cancer cells themselves, but rather the host stroma that produces the MMPs (Mott and Werb, 2004), suggesting

that the tumor actively interacts and communicates with its surrounding stroma, causing overexpression of numerous MMPs. These MMPs assist in tumor invasion by remodeling of the surrounding matrix, and in promotion of tumor growth in a network where a single MMP cleaves certain matrix components and activates other latent MMPs. Distinct MMPs are active during different stages of tumor development (Egeblad and Werb, 2002). When considering prognostic implications of MMP expression, the clinician should be aware that the host response to the tumor itself is able to provide important prognostic information. MMPs expressed by the tumor itself are also important for studying prognostic significance, as MMP expression is increased and strongly correlated with tumor invasiveness and poor prognosis reinforcing the concept that MMPs contribute to human cancer development (Agnantis *et al*., 2004; Lynch and Matrisian, 2002; Lochter *et al*., 1999; Folkman, 1995). In some cases, mouse models of tumor progression have complemented some of the data from human tumors regarding the role of MMPs in tumor progression (Bernhard *et al.*, 1994; Hua *et al.*, 1996, Kawamata *et al.*, 199). In fact, in experimental models of metastasis, the injection of tumor cells into the tail vein of mice deficient in MMPs resulted in fewer tumors growing in the animals relative to mice that were wild type for MMPs, complementing data showing decreased prognosis in cancer patients with elevated MMPs. Studies provide further evidence for the use of specific MMPs as prognostic factors (Nelson et al., 2000, Vihnen et al., 2002]. Research to identify MMPs as potential new biomarkers has shown that MMPs are very specific for types and stages of cancer (Egeblad and Werb, 2002; Liotta *et al*., 1980; Agnantis *et al*., 2004; Egeblad and Werb, 2002). MMPs can be useful for predicting tumor recurrence and metastasis risk (Vihnen and Kahari, 2002; Bode and Maskos, 2001; Egeblad and Werb, 2002, Vihnen and Kahari, 2002). Further complicating the story of MMPs as biomarkers is the data that show that not all MMPs are markers of poor prognosis and that sometimes, an augmented expression of MMPs correlates with better clinical results or treatment response (Kuittinen *et al*., 2002; Kuittinen *et al*., 1999; Nikkola *et al*., 2001, Scorilas *et al*., 2001). These

data point to the necessity of further careful studies in order to target detrimental MMPs without altering those that may actually provide better outcome to cancer patients (Brinckerhoff and Matrisian, 2002). In order to inhibit growth and invasion of cancer cells, many hypotheses have been taken into consideration to block MMP activity in the extracellular environment (Mitsiades *et al*., 2001). Studies using small molecule inhibitors of MMPs in early disease provide evidence that MMP inhibitors (MMPIs) would be useful as therapies to treat and prevent metastasis (Overall and Lopez, 2002). In this way, there may be the possibility for a new approach to cancer treatment in addition to traditional cytotoxic therapy.

Whether specific members of the MMP family are associated with oncogenesis of specific cancer types is still a matter of debate and varies among the tumor types and stage of lesions studied. However, the gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and associated with tumor aggressiveness, metastatic potential, and a poor prognosis. Therefore, these enzymes are suggested to be promising targets for the development of antitumor drugs. The involvement of these two enzymes in degrading type IV collagen which is a major component of the basement membrane indicates their contribution to metastasis. Number of studies has shown that MMP-9 levels are significantly elevated during the metastatic progression of malignant tumors since it requires proteolytic degradation of ECM components in basement membrane and stroma tissues. Elevated expression of MMP-9 is associated with increased metastatic potential in many cancer types including breast cancer, prostate cancer, brain cancer, melanoma and fibrosarcoma (Sehgal *et al*., 1996; Rao *et al*., 1993; Jones *et al*., 1999; Hujanen *et al*., 1994). MMP-9 was detected in 68% of primary breast carcinomas, either in the stromal compartment or adjacent to tumor cells (Jones *et al*., 1999). Furthermore, elevated serum levels of MMP-9 were shown to correlate with spontaneous metastasis in rat mammary tumor models (Nakajima *et al*., 1993). In addition, there is data to show that inhibition of MMP-9 expression and/or activity resulted in reduction of

in vitro as well as tumor invasion and metastasis in animal studies (Lozonschi *et al*., 1999; Garbisa *et al*., 1987; Davies *et al*., 1993). HT1080 cells, a human fibrosarcoma cell line have been used extensively as a model to study MMP-9/2 activity and expression. Similar to various other malignant tumors these cells express MMP-9 and MMP-2 at a higher level.

The MMP-2 promoter has a number of potential cis-acting regulatory elements (Qin *et al*., 1998). However, in recent years, NF-κB- dependent activation of MMP-2 has been reported (Kim and Koh, 2000). Studies on the promoter of MMP-9 have clearly identified that its transcription is mediated mainly via AP-1 transcription factor binding interactions. However, other reports on the promoter of MMP-9 suggest the involvement of NF-κB transcription factor for the activation of MMP-9 (Yoon *et al*., 2002).

Even though NF-κB and AP-1 transcription factors are regulated by different mechanisms, they appear to be activated simultaneously by the same multitude of stimuli (Abate *et al.,* 1990; Fan *et al.*, 2002). A number of reports have shown that these transcription factors appear to be regulated by the same intracellular signal transduction cascades. Indeed, the activation of mitogen activated protein kinases (MAPK) is often accompanied by the nuclear translocation of NF-κB, and many genes require the concomitant activation of AP-1 and NF-κB, suggesting that these transcription factors work cooperatively (Verma *et al.,* 1995). Three MAPK groups have been intensively studied: the extracellular signal-regulated kinases (ERKs) responsive to mitogens such as growth factors; the c-Jun N-terminal kinases (JNKs) and p38 MAPKs, which are activated by proinflammatory cytokines and environmental stresses (Whitmarsh and Davis, 1996, Minden and Carin, 1997). In tumors sustained MAPK activation could lead to enhance induction of proteolytic enzymes in the surrounding environment, leading to destruction of ECM a key histological marker of invasive carcinomas. In metastatic cells specifically ERK activity was shown to be higher when compared to nonmetastatic cancer cells (Panek *et al.*, 1997; Coutts and Murphy, 1998; McCawley *et al*., 1999; Adeyinka *et al*., 2002). In response to extracellular stimuli,

phosphorylated ERK translocates from cytoplasm to the nucleus and in the nucleus ERK activates a host of transcriptional factors including activating protein-1 (AP-1). Persistent activation of ERK in malignant cells can lead to enhanced induction of MMPs and this could lead to ECM and basement-membrane degradation allowing the cancer cells to invade into surrounding tissues and metastasize (Overall and Lopez, 2002; Egeblad and Werb, 2002).

3. Current and future developments

Matrix metalloproteinases are considered promising targets for cancer therapy due to their strong involvement in malignant pathologies, as their expression is up-regulated in such diseases and because they can degrade all components of the extracellular matrix. Preclinical studies analyzing MMP inhibition in tumor models brought positive results raising the idea that the development of strategies to inhibit MMPs may be a powerful tool to help defy cancer. Experimental models have been carried out to study the blocking of MMP gene transcription based on targeting extracellular factors, signal-transduction pathways or nuclear factors that activate expression of these genes.

Pleiotropic multiple mechanisms of inhibition of matrix metalloproteinases by biodrugs may be of significant importance to our understanding of the mechanism by which nutraceuticals elicit their antiangiogenic, anti-tumoral and anti-metastatic effects. The strong inhibition of gelatinolytic activities, the control of MMP gene expression and the antagonization of MMP activation by nutraceuticals may provide novel and plausible molecular mechanisms for how biodrugs obtained from natural dietary constituents may inhibit the growth and vascularization of rapidly proliferating neoplastic cells, through the additional support of substances created by Nature, with specific MMP inhibition activity useful in cytostatic therapies of the innovative treatment of cancer.

The naturally occurring inhibitors of MMP activity (TIMPs) were the first compounds to be considered for clinical development. Theoretically, the ability of TIMPs to potently and specifically inhibit the activity of several MMPs could

result in a beneficial therapeutic effect. However, the lack of effective methods of systemic gene delivery has limited the clinical utility of this approach, whereas the development of synthetic inhibitors of MMPs has been actively pursued and widely tested in clinical trials. Inhibitors of MMPs fall into three pharmacologic categories: 1) collagen peptidomimetics and nonpeptidomimetics, 2) tetracycline derivatives, and 3) bisphosphonates. Problems with the peptidic MMP inhibitors include poor oral bioavailability (except for marimastat) and a relative lack of specificity for the MMPs thought to contribute to cancer progression. In an effort to avoid these problems, several nonpeptidic MMP inhibitors have been rationally synthesized on the basis of the three-dimensional x-ray crystallographic conformation of the MMP active site. Several of these molecules demonstrated antitumor activity in preclinical models and were selected for clinical development. The rational chemical design of MMP inhibitors made possible the synthesis of compounds with specific inhibitory activity against the MMP subtypes that predominate in certain diseases, such as cancer and arthritis.

The earliest MMP inhibitors were peptide derivatives designed from the knowledge of the amino acid sequence of collagen at the site of cleavage by collagenase. With the development in the field, medicinal chemists focused away from the peptidic inhibitors because of the difficulties in obtaining good oral activity and lack of specificity for MMPs. As a result, a number of non-peptidic inhibitors have been discovered by high-throughput screening of natural product and also potent MMP inhibitors have been synthesized with desirable chemical functional groups.

Among known MMP inhibitors doxycycline, a tetracycline derivatives has reported to be involved in MMP inhibition via a number of mechanisms. It has a potency to inhibit both the collagenases, MMP-1, -3, and -13, and the gelatinases, MMP-2 and -9, via mechanisms including 1) blocking the activity of mature MMPs by chelation of the zinc atom at the enzyme binding site, 2) interfering with the proteolitic activation of pro-MMP into their active form, 3) reducing the expression of MMPs, and 4) protecting MMPs from proteolytic and oxidative

degradation. Doxycycline has been evaluated in preclinical cancer models and has entered early clinical trials in patients with malignant diseases. According to reported data, these tetracycline derivatives inhibit not only the activity but also the production of MMPs and are suggested for the treatment of disorders in which the MMP system becomes amplified, such as degenerative osteoarthritis, periodontitis, and cancer.

As a material which can exert anticancer effects in a highly metastatic cell line melanoma, its worth to study capability of QAGlc to act against metastasis by blocking the cellular invasiveness of cancer cells. For that, assessment of its effect on MMP's is important because cancer cells invade adjacent tissues via production of matrix metalloproteinases. More specifically, to speculate ability of QAGlc to act against metastasis, MMP-2 and MMP-9, MMP's that have been reported to be greatly involved in tumor progression were selected as targets. Therefore, this part of the research was designed to test the effects of QAGlc on MMP2/9 activity and expression in HT1080, human fibrosarcoma cell line.

Materials and Methods

1. Materials

HT1080 cells were obtained from American Type of Culture Collection (Manassas, VA, USA). All the materials required for culturing of cells including cell culture media were purchased from Gibco BRL, Life Technologies (USA). MTT reagent, gelatin, agarose, doxycycline, PMA (phorbol 12-myristate 13-acetate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. Cell culture

Human fibrosarcoma cells (HT1080) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin-streptomycin. For experiments, cells were detached with trypsin-EDTA and plated onto 24- or 96-well plates at a plating density of 7×10^5 and 1.5×10^5 per well, separately.

3. Cytotoxicity determination of QAGlc in fibrosarcoma cells

Cell viability was evaluated with the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method which measures conversion of tetrazolium salt in to blue formazan by viable cells as described by Hansen *et al.* (1989). Briefly, Equal number of HT1080 cells (4000 cells per well) cultured in 96-well plates with serum and serum free media were treated with various concentrations of QAGlc for 24 h and for 48 h. After the incubation period, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, DMSO (100 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the optical density (OD) at 540 nm using GENios® microplate reader (Tecan Austria GmbH, Austria).. Relative cell viability was determined by the amount of MTT converted to formazan salt. The percentage of viable cells compared to the control was

calculated as (OD of treated cells − OD of blank / OD of control − OD of blank) ×100 and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $P<0.05$ was considered significant. Nontoxic concentrations (below 10% cell death), of tested sample were selected to carryout related assay procedures.

4. Gelatin zymography

MMP-9 and MMP-2 activities in HT1080 cells was assayed by gelatin zymography as described previously.[12] For this, approximately $2x10^5$ cells/mL HT1080 cells in serum free DMEM medium were seeded in 24-well plates and pre-treated with different concentrations of QAGlc and Glc for 1 h. MMP expression was stimulated by treatment of PMA (10 ng/mL) and cells were cultured for 48 h. Cell conditioned medium was subjected to substrate gel electrophoresis. Similar amount of protein containing conditioned media were applied without reduction to a 10% (w/v) polyacrylamide gel impregnated with 1 mg/mL gelatin. After electrophoresis, gel was washed in 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 at room temperature and incubated overnight at 37°C in zymography developing buffer containing 10 mM $CaCl_2$, 50 mM Tris-HCl, and 150 mM NaCl. The gel was then stained with a solution of 0.1% Coomassie Blue. In this assay clear zones against the blue background indicate the presence of gelatinolytic activity. The image of the gel was recorded using Fujifilm Image Reader LAS-3000 software in Science Image System, LAS 3000 (Fujifilm Life Science, Tokyo, Japan). The gelatinolytic activities were quantified using Multi Gauge V3.0 software.

To assess direct inhibitory activity against the enzyme present in the supernatant a separate experiment was conducted. Firstly, HT 1080 cells were treated with PMA (10 ng/mL) and incubated for 3 days to obtain conditioned media containing MMP's. These conditioned media was then treated with similar concentrations of doxycycline and QAGlc and incubated for 1 h. The reaction mixture was then zymographed according to the same above procedure.

5. Reporter gene assay for MMP-9, MMP-2, AP-1 and NF-κB

HT1080 cells cultured in 10 cm culture dishes were transiently transfected with MMP-9 promoter containing pGL3 luciferase reporter vector (Promega, Madison, WI), AP-1 binding site containing luciferase reporter plasmid (Colontech, Palo Alto, Canada) and NF-κB promoter containing luciferase reporter vector by Lipofectamine™ 2000™ reagent (Invitrogen). Beta-galactosidase expression vector was co-transfected with the reporter vector to serve as an internal control of transfection efficiency. Transfected cells sub-cultured in 24-well plates were treated with different concentrations of QAGlc. Following stimulation with PMA cells were cultured for 24 h. Cells were washed once with cold PBS and lysed with 200 μl/well lysis buffer (25 mM Tri-HCl, pH 8.0, containing 2 mM DDT and 1% Triton-X 100). Aliquots of cell lysate and luciferase substrate (Promega) were mixed in equal amounts in a 96-well plate and luminescence intensity was measured with a luminescence microplate reader (Tecan Austria GmbH, Austria). Beta-galactosidase activity was measured with ONPG buffer. The level of reporter gene expression was determined as a ratio, compared with cells stimulated by PMA (10 ng/ml) alone and represented as relative luciferase activity.

Transfection efficiency was determined by X-Gal staining method. Briefly, transfected cells were fixed with 0.5% glutaraldehyde and stained with X-Gal solution containing 20 mM $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and 1 mM $MgCl_2$. After 24 h of incubation at 37°C, transfected cells were visualized with blue color under a light microscope.

6. Extraction of nuclear and plasma protein

For separate extraction of nuclear and cytoplasmic proteins, CelLytic™ NuCLEAR™ Extraction kit (S26-36-23, Sigma-Aldrich Co., MO, USA) was used following manufacturer's instructions. Briefly, cells treated with QAGlc were collected and lysed with 0.5 ml of lysis buffer (500 μl, hypotonic lysis buffer, 5 μl, 0.1 M DTT, 5 μl protease inhibitor cocktail) and incubated for 15 min on ice.

Igepal CA-630 solution (36 μl) was added and vortex for 20 seconds. Nuclei were separated by centrifugation at 13,000 × g and supernatant (cytoplasmic protein) was collected. Precipitated nuclei were lysed with 70 μl of extraction buffer mix (98 μl, extraction buffer, 1 μl of 0.1 M DTT and 1 μl protease inhibitor cocktail) for 30 min and nuclei protein were collected by centrifugation at 13,000 × g.

7. Western blot analysis

After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Briefly, harvested cells were washed twice with ice-cold PBS; resuspended in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) containing antipain (1 μg/ml), aprotinin (1 μg/ml), chymostatin (1 μg/ml), leupeptin (0.1 μg/ml), pepstatin (1 μg/ml), and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and incubated on ice for 20 min. Supernatants were recovered by a 10-min centrifugation (12,000 x *g*) at 4 °C, and protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin as a standard. Proteins (20-40 μg) were diluted in 5x sample buffer (10% SDS and 100 mM each dithiothreitol, glycerol, bromphenol blue, and Tris-HCl) and resolved in 4-20% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane. Then proteins were transferred onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany), and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with the appropriate dilution of primary antibodies (1:500 dilutions) related to apoptosis and cell cycle progression. After three 5-min washes with Tris-buffered saline and 0.1% Tween 20, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. They were then washed again three times with Tris-buffered saline and 0.1% Tween 20, rinsed briefly with PBS, and developed with enhanced chemiluminescence reagents

(ECL, Amersham Biosciences, UK). Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). Detection of β-actin (1:5000 antibody dilution) was used as control for loading of equal amount of protein.

8. Cell invasion and mobility assay

Invasiveness of HT1080 cells through the ECM was assessed using CHEMICON® Cell Invasion Assay Kit (ECM550, Chemicon International Inc., CA, USA) according the instructions. Briefly, HT1080 cells were seeded into 24-well plate inserts bottom covered with reconstituted basement membrane matrix proteins coated polycarbonate membranes, at a density of 1.0 × 105 cells/ml using serum-free media. Cells were pretreated with determined concentrations of CCOS-3 for 1 h and MMP expression was stimulated by PMA (10 ng/ml). After incubation for 36 h in a cell culture incubator, non-invasive cells were carefully removed using a cotton-tipped swab without damaging the polycarbonate membrane. Cells invaded through the basement membrane matrix protein layer and attached to the bottom of polycarbonate membrane were stained for 20 min with the staining solution. Membranes were rinsed thoroughly with water to remove excess staining solution and stained cells were dissolved in 10% acetic acid (200 µl/membrane). Cells were then quantified by colorimetric reading of optical density at 560 nm.

9. Statistical analysis

Results are presented as mean ± standard error of the mean (n = 3). Student’s t-test was used to determine the level of significance.

Results and Discussion

1. Inhibition of MMP-9 and MMP-2 expression in QAGlc treated HT1080 cells assessed by gelatin zymography

To analyze the inhibitory effects of QAGlc on MMP-9 and MMP-2 activity and expression, zymography experiment was performed using conditioned media obtained from human fibrosarcoma cell line, HT1080. In order to stimulate MMP expression levels in these cells, cell medium was stimulated with phorbol myristate acetate (PMA) which is a potent tumor inducer. HT1080 cells express MMP-9 and MMP-2 to a substantial level in the presence of PMA. When PMA was administered to HT1080 cells at 10 ng/ml concentration, MMP-9 and MMP-2 activities were increased by approximately 60% and 90% respectively (Fig. 1). Lytic zones resulted due to gelatinolytic activities of MMP-9 and MMP-2 were used to evaluate the QAGlc mediated inhibition of MMP-9/MMP-2 expression and activation. Size and the intensity of lytic zones were greatly reduced in the presence of QAGlc and it clearly indicated that MMP-9 and MMP-2 activities in HT1080 cells were markedly inhibited in the presence of QAGlc. Moreover, inhibitory effect of QAGlc on MMP-9 and MMP-2 showed a concentration dependant pattern and at concentration 60 μg/ml, QAGlc showed approximately 50% inhibition of MMP-9 led gelatinolytic activity. This activity was much clear than the effect of doxycycline (at 10 μg/ml concentration), a tetracycline analogue we used in this study as the positive control to compare the inhibitory effects. Further, at the same concentration of QAGlc, about 62% inhibition of MMP-2 expression level was observed in the zymogram. In this study we carefully selected the non toxic concentrations of QAGLC. Therefore, the data confirmed that cytotoxicity did not contribute to the observed MMP-9 and MMP-2 inhibitory potentials of QAGlc.

It is important to know whether QAGlc act as a direct inhibitor of gelatinases (MMP-9 and MMP-2) as many of the other synthetic inhibitors do. Therefore PMA-stimulated conditioned media was treated with QAGlc and incubated for

one hour. Separate experiment was conducted by treating conditioned media with doxycycline. Once the zymography was conducted following the same procedure, any alterations in enzymatic activities of MMP-9 or MMP-2 could not be observed in QAGlc treated groups (Fig. 2). These results suggested that QAGlc did not have a direct enzyme inhibition potential. In the presence of similar concentrations of doxycycline, a slight decrement in enzymatic activities was observed. This lytic zone reduction was only due to direct inhibition of enzyme activity but not due to inhibition of gene expression. These data are in agreement with that of other research on doxycycline that this material has a capability to directly inhibit MMP's via mechanisms involving chelation.

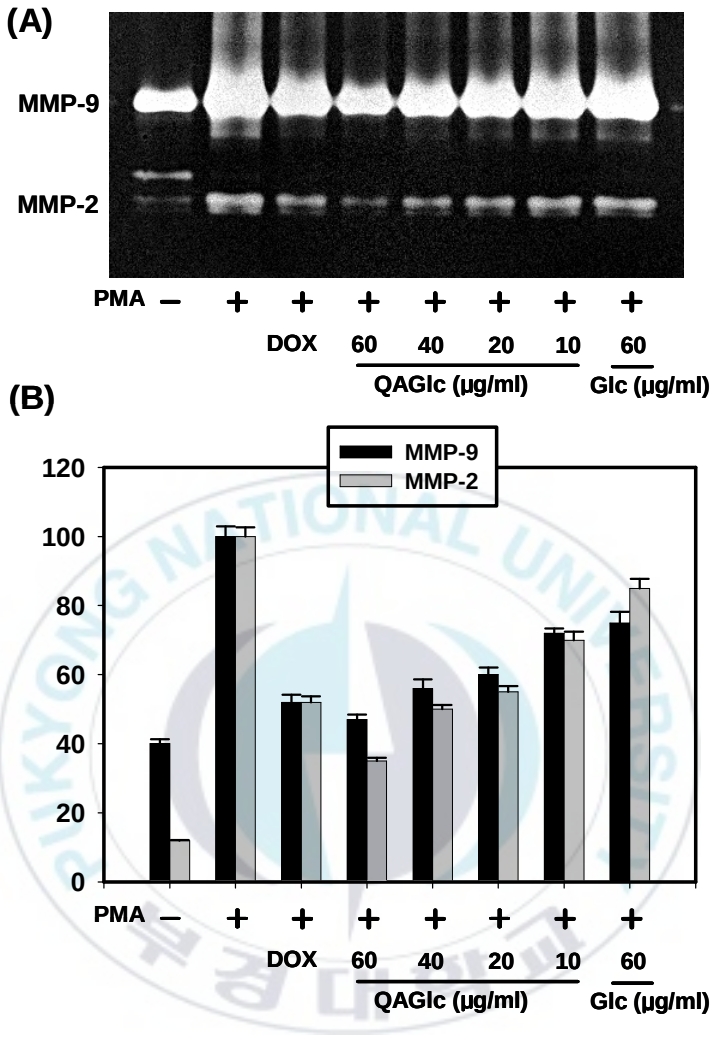

Fig.1. MMP-9 and MMP-2 gelatinolytic activities in PMA stimulated HT1080 cells in the presence of QAGlc. Cells were pre-treated with test compounds for 1 h in the presence of serum free medium and stimulated with PMA. The conditioned media was then subjected to electrophoresis in a 10% (w/v) polyacrylamide gel impregnated with gelatin. Zymogram developed in the presence of developing buffer was stained and the image of the gel was recorded using an image reader. DOX: Doxycycline

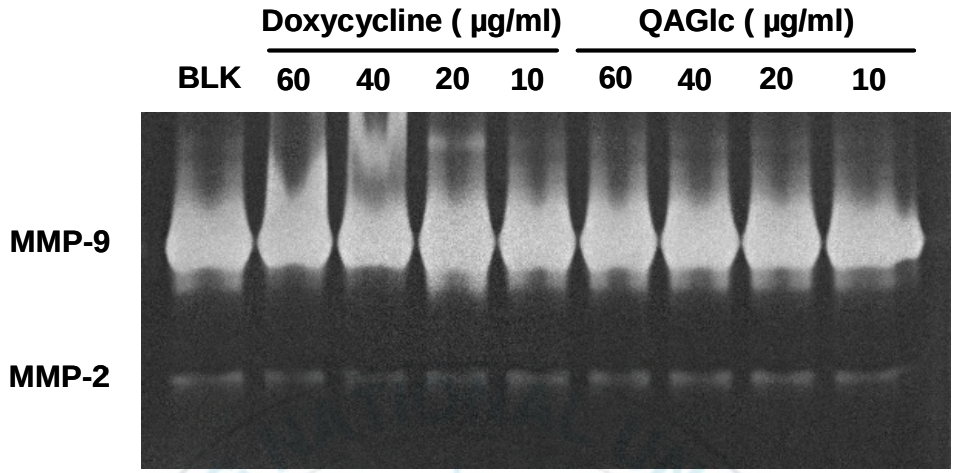

Fig.2. MMP-9 and MMP-2 gelatinolytic activities in PMA stimulated HT1080 cells in the presence of QAGlc and doxycycline. HT 1080 cells were treated with PMA and incubated for 3 days to obtain conditioned media containing MMP's. This was then treated with doxycycline and QAGlc and incubated for 1 h. The conditioned media was then subjected to electrophoresis in a 10% (w/v) polyacrylamide gel impregnated with gelatin. After electrophoresis, renaturation buffer was added and gel was incubated for 24 h following addition of developing buffer. The gel was destained following staining. The image of the gel was recorded using Fujifilm Image Reader. The gelatinolytic activities were quantified using Multi Gauge V3.0 software. BLK: non-treatment group.

2. Assessment of transcriptional regulation of MMP-9 and MMP-2 following treatment with QAGlc

To extend these observations to interactions within the context of transcriptional regulation of MMP-9 and MMP-2, we assessed the effect of QAGlc on promoter activity of MMP-9 and MMP-2. For that luciferase reporter vectors containing MMP-9 and MMP-2 promoters were transfected to HT1080 cells in separate experiments. A high (about 4-fold) luciferase activities were noted in both in MMP-9 and MMP-2 transfected cells when stimulated with PMA (Fig. 3 and 4). However, the luminescence reading values obtained for MMP-2 were greatly lower than that of MMP-9. These results were well correlated with zymography that lytic zones resulted from MMP-9 were much clear than that resulted from MMP-2. However, the promoter activities of MMP-9-luciferase reporter gene and MMP-2-luciferase reporter gene were inhibited nearly 3-fold when the cells were treated with QAGlc at 60 μg /ml concentration. And both MMP-9 and MMP-2 inhibitions followed a dose-dependant pattern.

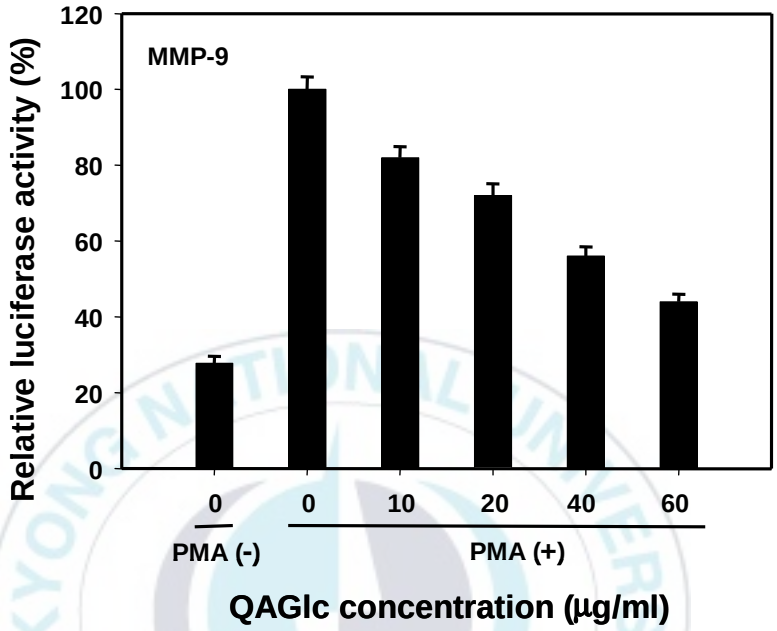

Fig.3. Suppression of MMP-9 gene promoter activity in QAGlc treated HT1080 cells. Cells were co-transfected with MMP-9-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with PMA. Luciferase enzyme activity that represents MMP-9 promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

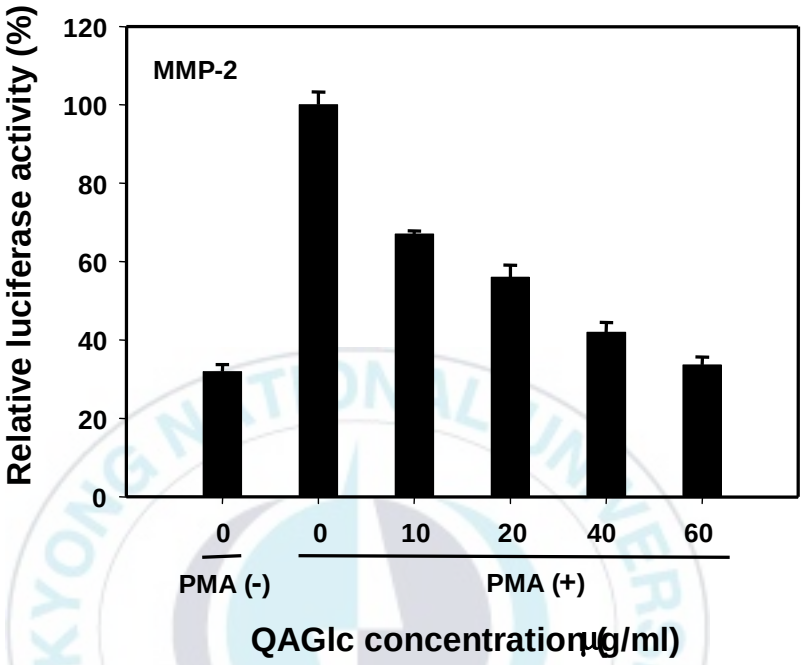

Fig.4. Suppression of MMP-2 gene promoter activity in QAGlc treated HT1080 cells. Cells were co-transfected with MMP-2-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with PMA. Luciferase enzyme activity that represents MMP-2 promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

3. Assessment of transcriptional regulation of NF-κB and AP-1 following treatment with QAGlc

In order to test whether the suppression of MMP-9/2 promoter activities had any correlation to regulation of AP-1 and NF-κB promoter activities, reporter gene assay was conducted using AP-1 and NF-κB promoter containing luciferase reporter vectors. These vectors were first transfected to HT1080 cells in two separate experiments and then the transfected cells were stimulated with PMA. When the cells were transfected with NF-κB promoter containing luciferase reporter vector, luciferase activity in PMA stimulated group was increased by approximately 3-fold whereas about 65% inhibition was observed once the cells were treated with QAGlc at 60 μg/ml concentration (Fig. 5 and 6). And the suppression followed a clear dose-dependant pattern. A more or less similar pattern was observed when the cells were transfected with AP-1 promoter containing luciferease vector followed by treatment with QAGlc. Further, in the presence of the highest concentration of QAGlc (60 μg/ml) that employed in this study, about 60% inhibition of AP-1 promoter activity was observed. Taken together, these experiments suggest that QAGlc suppresses MMP-9 and MMP-2 expression via down regulation of AP-1 and NF-κB. To further confirm the effects of QAGLc on MMP-9/MMP-2 by extending the experiment to protein expression level, QAGlc treated cells were stimulated with PMA and cell lysates were obtained to analyze employing western blot experiment. A clear concentration dependent suppression effect was observed in MMP-2/MMP-9 protein levels when cells were treated with QAGlc (Fig. 7). In case of MMP-2, protein amounts in both active and inactive forms were greatly suppressed in the presence of QAGlc. However, under experimental conditions employed, only active form of MMP-9 was observed and it was greatly reduced in the presence of QAGlc.

Moreover, western blot analysis carried out to quantify the NF-κB (p65) protein expression levels in both cytosol and nucleus following treatment of QAGlc, confirmed that QAGlc is involved in suppression of activity and translocation of NF-κB (Fig. 8). However, NF-κB translocation and expression

was not changed in the presence of doxycycline. But this effect of QAGlc was much clear and followed a concentration-dependent pattern. This suppression of NF-κB subsequently lead to MMP-9 and MMP-2 inhibition Therefore, this observation further strengthened the MMP-2 and MMP-9 inhibitory potential of QAGlc assessed employing zymography.

In order to test whether TIMP1 and TIMP-2, the natural MMP-2 and MMP-9 inhibitors respectively are altered in the presence of QAGlc, their protein expresseion levels were assessed. However, no any significant differences in TIMP-1 and TIMP-2 protein levels were observed in the presence of QAGlc even at relatively higher concentrations.

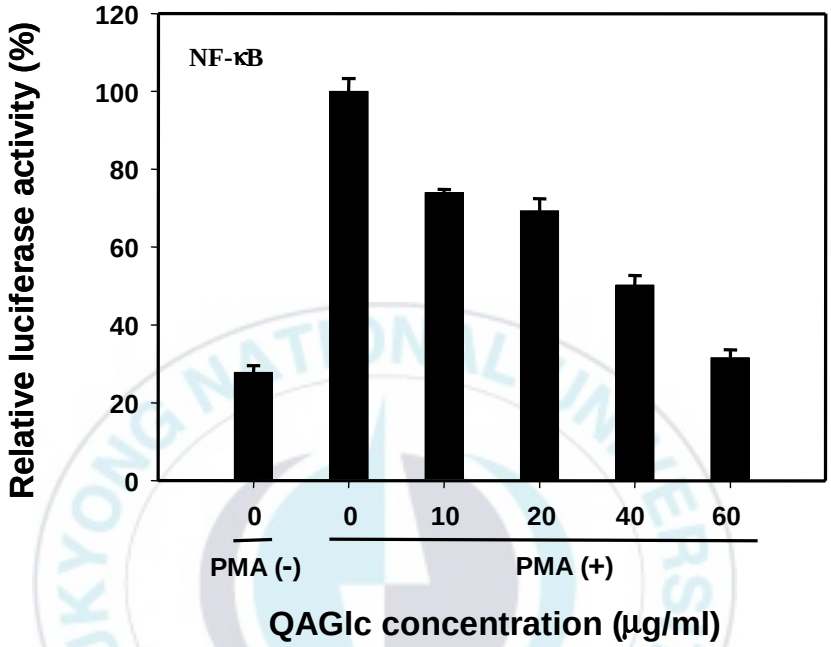

Fig.5. Suppression of NF-κB gene promoter activity in QAGlc treated HT1080 cells. Cells were co-transfected with NF-κB-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with PMA. Luciferase enzyme activity that represents NF-κB promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

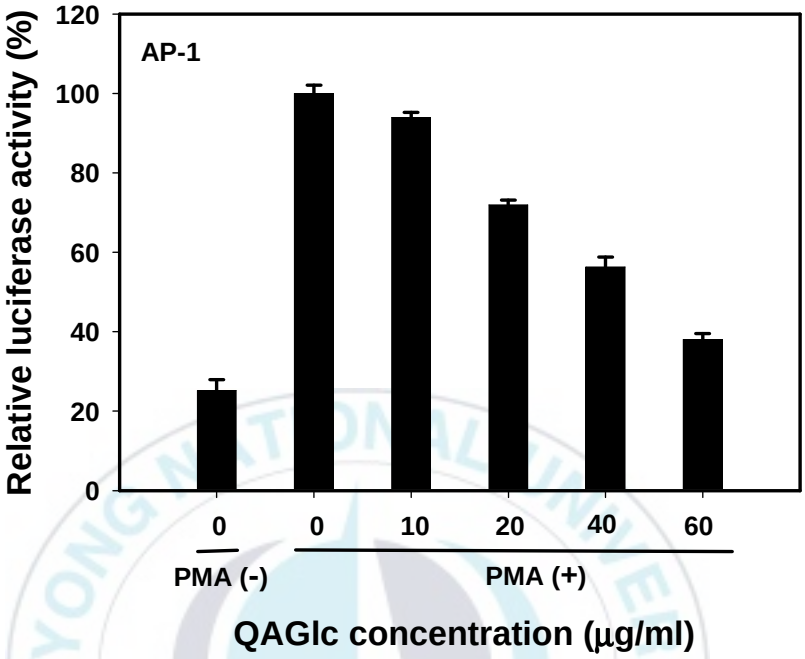

Fig.6. Suppression of AP-1 gene promoter activity in QAGlc treated HT1080 cells. Cells were co-transfected with AP-1-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with PMA. Luciferase enzyme activity that represents AP-1 promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

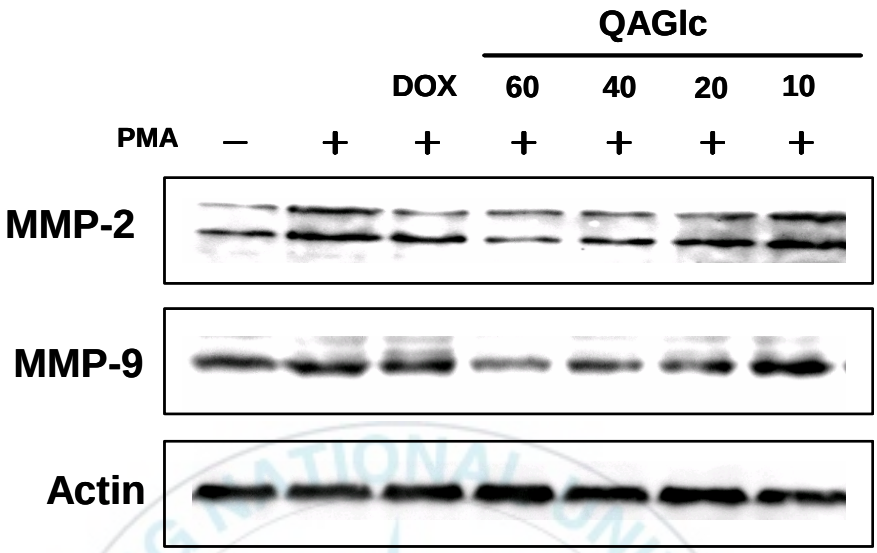

Fig. 7. Western blot analysis of MMP-2 and MMP-9 protein expressions in HT1080 cells treated with QAGlc. After treatment of QAGlc cells were stimulated with PMA. Whole cell extracts were then obtained and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; DOX: Doxycycline.

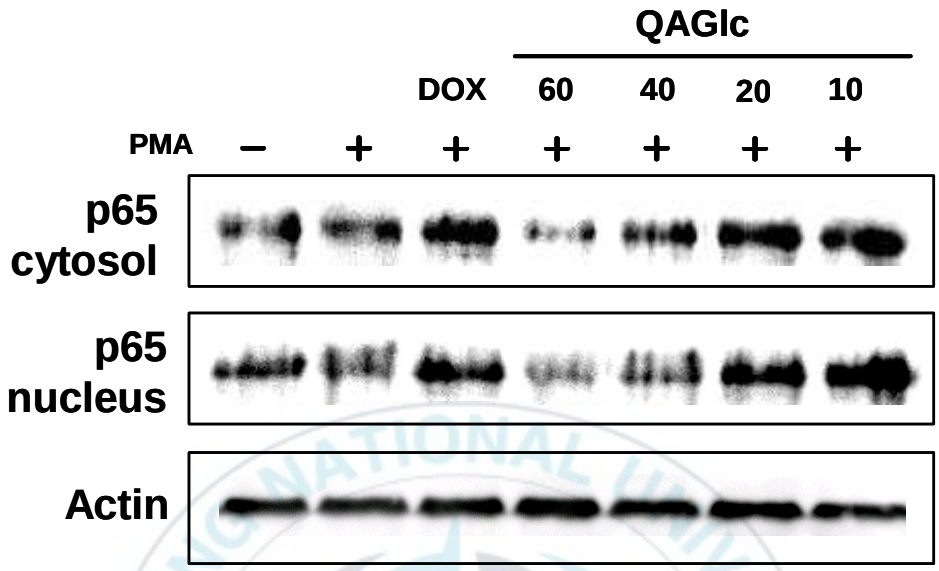

Fig. 8. Western blot analysis of NF-κB (p65) protein expressions in HT1080 treated with QAGlc. After treatment of QAGlc cells were stimulated with PMA. Proteins present in neucleus and cytososl were then extracted and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; DOX: Doxycycline.

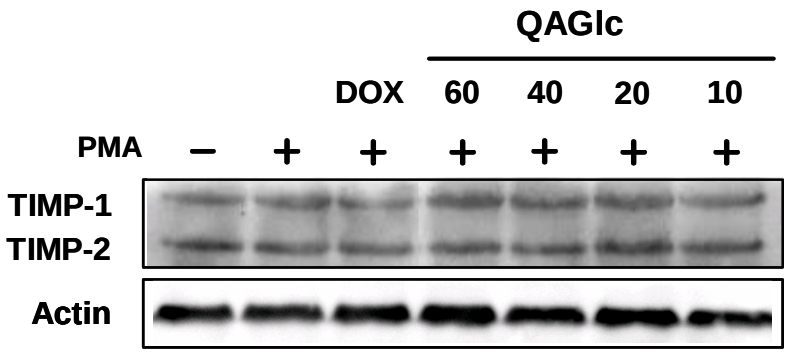

Fig. 9. Western blot analysis of TIMP-1 and TIMP-2 protein expressions in HT1080 cells treated with QAGlc. After treatment of QAGlc cells were stimulated with PMA. Whole cell extracts were then obtained and resolved by denaturing SDS-PAGE. Proteins were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were then transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; DOX: Doxycycline.

4. Inhibition of invasiveness of HT1080 cells following treatment with QAGlc

In order to examine the potential influence of QAGlc treatment on the invasiveness of the HT1080 cells, cell invasion assay was performed in an invasion chamber. As shown in Fig. 10, treatment with PMA greatly enhanced the invasion of HT1080 cells through the ECM layer. However, in the presence of 20 ug/ml concentration of QAGlc, about 50% of the invasion was decreased after 48 h. The migration of HT1080 cells were blocked about 70% without affecting the cell viability when QAGlc concentration was increased upto 60 ug/ml. Therefore the strong inhibitory effect of QAGlc on the invasion was suggested to be related to its higher potential to inhibit MMP gene expression.

5. Inhibition of mitogen-activated protein kinase pathway in HT1080 cells following treatment with QAGlc

In order to test whether QAGlc has a potency to act against members in the MAPK pathway that generally involved in the regulation of gelatinases via NF-kB and AP-1, their protein expression levels were assessed using western blot analysis. QAGlc exerted inhibitory effects on main MAP kinases including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38MAPK that stimulated with PMA (Fig. 11). Therefore it can be presumed that inhibition of MAPK contributes to the inhibition of MMP-9 and MMP-2 expression via suppression of NF-kB and AP-1. Moreover, findings of this study present the potency of QAGlc to alter both AP-1 and NF-kB expressions. So it's possible to presume that both these activities are contributing to its inhibitory potential of MMP-9. However studies have come up with the findings that, the response to AP-1 is strikingly enhanced when NF-κB subunits are present and vice versa (Stein *et al.*, 1993). On the basis of these collective findings, one could speculate, therefore, an intriguing possibility that NF-κB and AP-1 may modulate the activity of each other, thus expanding the scope of these two rapidly inducible transcription factors. One clue to the interactions between the AP-1 and NF-κB activation pathways was the finding that activation of the MAPK pathway leads to

the activation of JNK and NF-κB (Tapon *et al.,* 1998). Therefore, the inhibitory effects of QAGlc on MMP-2 and MMP-9 can be speculated to be regulated via suppression of both NF-κB and AP-1.

To our knowledge, there are no synthetic MMP inhibitors identified so far having capability to inhibit MMP transcriptional regulation. Compounds that can exert an effect at transcriptional level could be expected to have a promising potential to develop potent inhibitors. That is because they can interfere with the up-regulation of MMP-9 and MMP-2 expression and thereby can remarkably decrease the total MMP activity. Therefore QAGlc represents a potential gelatinase expression inhibitor in HT1080, human fibrosarcoma cells.

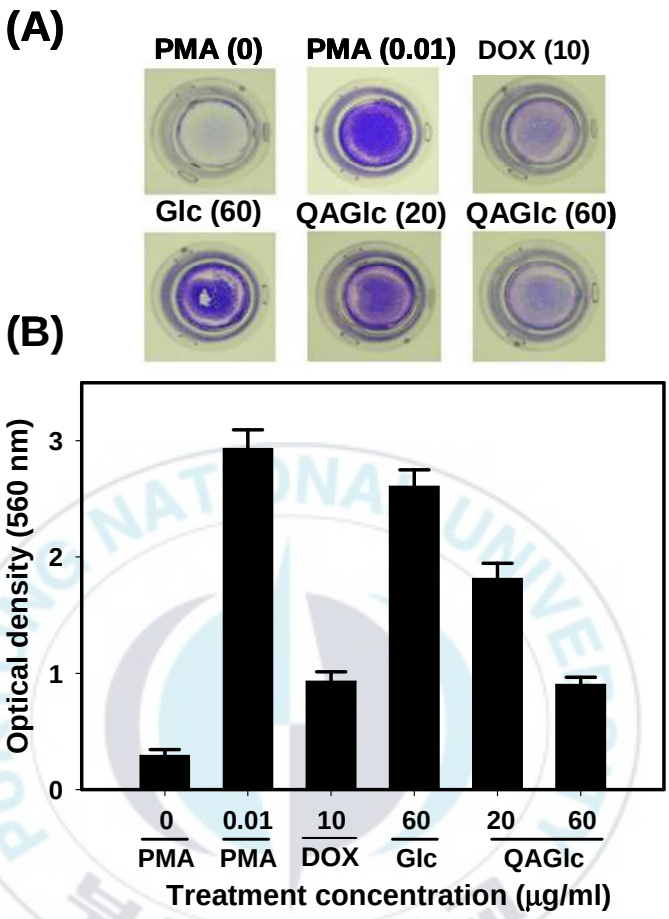

Fig. 10. Cell invasion assay in HT1080 cells. Cells growing in invasion chambers were treated with test compounds and stimulated with PMA. Cell invasion through ECM containing basement membrane was analyzed after 48 h by staining the cells that invaded the lower surface of the membrane. A: stained cells that invaded through the membrane in different invasion chambers conducted with different treatment groups. B: Optical density resulted from the stained cells and expresses the amount of cells invaded. DOX: Doxycycline.

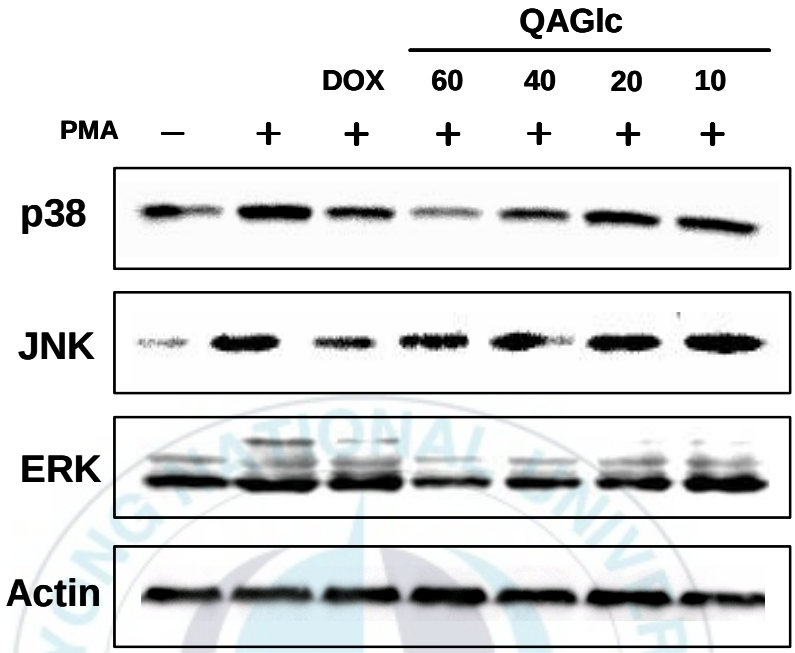

Fig. 11. Western blot analysis of ERK, JNK and p38 protein expressions in HT1080 cells treated with QAGlc. After one hour of treatment of QAGlc, cells were stimulated with PMA. Whole cell extracts were then obtained and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. BLK: non-treatment group; DOX: Doxycycline.

6. Summary

In the present research, an effort was taken to identify effect of newly synthesized glucosamine derivative having quaternized amino functionality on activity and expression of MMP-2/MMP-9 levels. Results exhibited that QAGlc had a potency to alter promoter activities of MMP-2, MMP-9, AP-1 and NF-kB in transfected cells stimulated with PMA. These effects on MMP-9 and MMP-2 were further observed in zymography experimental results. Reduction in activity and translocation of NF-kB in the presence of QAGlc linked its ability to inhibit both MMP-9 and MMP-2 via down regulation of NF-kB. Further, results of this study suggested that effect of QAGlc on AP-1 also contributed to the ability of QAGlc to inhibit MMP-9. However, protein expression levels in natural inhibitors of MMP-2 andMMP-9, TIMP-1 and TIMP-2 respectively were not altered in the presence of QAGlc. In addition, inhibitory potency of QAGlc on invasiveness of PMA stimulated HT1080 cells confirmed its ability to act against two major gelatinases, MMP-2 and MMP-9. Moreover, suppression of protein levels in ERK, JNK and p38 suggested a link between inhibitions of MAPK's in the MMP-9 inhibitory potential via down regulation of AP-1. Taken together, the results of this study present a novel potent inhibitor of MMP-9 and MMP-2 that act via down regulation of AP-1 and NF-kB.

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Part 3

Inhibition of NF-κB Pathway in RAW264.7, Mouse Macrophage Cells by Quaternized Amino Glucosamine

Introduction

1. Background of nuclear factor κB (NF-κB)

Recent advances in scientific understanding of the mediators involved in acute and chronic inflammatory diseases and cancer have led to new strategies in the search for effective therapeutics. Moreover, breakthroughs in the elucidation of regulatory mechanisms involved in the transcription and translation of a variety of mediators have led to increased interest in therapeutic approaches directed at the level of gene transcription (Albert, 2001; Yamamoto *et al.*, 2001). Number of studies directed towards this approach strongly supports the hypothesis that NF-κB is a functionally relevant target of treatment of number of disease occurrences including cancer. Nuclear factor κB (NF-κB) belongs to a family of closely related dimeric transcription factor complexes composed of various combinations of the Rel/NF-κB family of polypeptides. The family consists of five individual gene products in mammals, RelA (p65), NF-κB1 (p50/pl05), NF-κB2 (p49/pl00), c-Rel, and ReIB, all of which can form hetero-or homodimers. These proteins share a highly homologous 300 amino acid"Rel homology domain" which contains the DNA binding and dimerization domains. At the extreme C-terminus of the Rel homology domain is a nuclear translocation sequence important in the transport of NF-κB from the cytoplasm to the nucleus. In addition, p65 and cRel possess potent transactivation domains at their C-terminal ends (Yamamoto *et al.*, 2001).

The activity of NF-κB is regulated by its interaction with a member of the inhibitor IκB family of proteins (Baldwin *et al*., 1996). This interaction effectively blocks the nuclear localization sequence on the NF-κB proteins, thus preventing migration of the dimer to the nucleus. A wide variety of stimuli activate NF-κB through what are likely to be multiple signal transduction pathways. Included are bacterial products (LPS), some viruses (HIV-1, HTLV-1), inflammatory cytokines (TNFa, IL-1), environmental and oxidative stress and DNA damaging agents. Apparently common to all stimuli however, is the phosphorylation and subsequent

degradation of IκB. The phosphorylation of the IκB proteins is a key step involved in the regulation of Rel/NF-κB complexes. The phosphorylation of the IκB proteins is mediated by IκB kinases (IKKs) (Zandi *et al.,* 1999), whose activity is strongly induced by activators of the NF-κB pathway. IKK activity is present in a high-molecular-weight complex containing at least two kinase subunits, IKKα and IKKβ, and the associated modulatory protein, IKKγ. Biochemical analysis and gene disruption studies of the IKK genes in mice has indicated that IKKβ is the critical kinase involved in activating the NF-κB pathway, while IKKα likely plays an accessory role (Zandi *et al.,* 1999). The activated IKK complex phosphorylates the IκB proteins on two closely spaced serine residues in the amino terminus of these proteins. Phosphorylation of IκB leads to its ubiquitination on two amino-terminal lysine residues by the E3 ubiquitin ligase complex, thus targeting it for degradation (Zandi *et al.,* 1999). Freed of their association with the IκB subunits, the NF-κB proteins translocate to the nucleus, where they bind to specific elements in the promoter regions of target genes to activate gene expression.

2. Involvement of NF-κB in the occurrence of diseases including cancer

It is well-known that NF-κB plays a key role in the regulated expression of a large number of pro-inflammatory mediators including cytokines such as TNF, IL-1, IL-6, and inducible nitric oxide synthase (iNOS) (Baldwin, 1996; Pahl, 1999; Gerondakis *et al.,* 1998; Ghosh *et al.,* 1998). Such mediators are known to play a role in the recruitment of leukocytes at sites of inflammation and in the case of iNOS, may lead to organ destruction in some inflammatory and autoimmune diseases. The importance of NF-κB in inflammatory disorders is further strengthened by studies of airway inflammation including asthma, in which NF-κB has been shown to be activated. This activation may underlie the increased cytokine production and leukocyte infiltration characteristic of these disorders. In light of the recent findings with regard to glucocorticoid inhibition of NF-κB, one may speculate that these effects are mediated through an inhibition of NF-κB.

Further evidence for a role of NF-κB in inflammatory disorders comes from studies of rheumatoid synovium (Tak *et al.,* 2001). Although NF-κB is normally present as an inactive cytoplasmic complex, recent immunohistochemical studies have indicated that NF- κB is present in the nuclei, and hence active, in the cells comprising rheumatoid synovium. Furthermore, NF-κB has been shown to be activated in human synovial cells in response to stimulation with TNF-α or IL-1 (Roshak, *et al.,* 1996). Expression of IKK has been shown in synoviocytes of rheumatoid arthritis patients and gene transfer studies have demonstrated the central role of IKK-P in stimulated inflammatory mediator production in these cells (Aupperele *et al.,* 1999; Aupperle *et al.,* 2001). More recently, the intra-articular administration of a wild type IKK-P adenoviral construct was shown to cause paw swelling while intra- articular administration of dominant-negative IKK-P inhibited adjuvant-induced arthritis in rat (Tak *et al.,* 2001).

The NF-κB/Rel and IκB proteins are also likely to play a key role in neoplastic transformation and metastasis (Gilmore *et al.,* 1996; Rayet *et al.,* 1999). These family members are associated with cell transformation in vitro and in vivo as a result of overexpression, gene amplification, gene rearrangements or translocations. In addition, rearrangement and/or amplification of the genes encoding these proteins are seen in certain human lymphoid tumors. In addition, a role for NF-κB in the regulation of apoptosis has been reported, strengthening the role of this transcription factor in the regulation of tumor cell proliferation. TNF, ionizing radiation and DNA damaging agents have all been shown to activate NF-κB which in turn leads to the up-regulated expression of several anti-apoptotic proteins. Conversely, inhibition of NF-κB has been shown to enhance apoptotic-killing by these agents in several tumor cell types.

As this likely represents a major mechanism of tumor cell resistance to chemotherapy, inhibitors of NF-κB activation may be useful chemotherapeutic agents as either single agents or adjunct therapy. Recent reports have implicated NF-κB as an inhibitor of skeletal cell differentiation as well as a regulator of cytokine-induced muscle wasting (Guttridge *et al.,* 2000) further supporting the

potential of NF-κB inhibitors as novel cancer therapies. NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The Rel/ NF-κB family of transcription factors are involved mainly in stress-induced, immune, and inflammatory responses. In addition, these molecules play important roles during the development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures.

Evidence that inhibition of NF-κB in cells induced to express an oncogenic form of Ras or in some cancer cell lines led to an apoptotic response (Mayo *et al.*, 1997) raised the possibility that inhibitors of NF-κB may function as stand-alone cancer therapies or as cancer-preventive compounds (Madrid *et al.*, 2000). Regarding the first hypothesis, it is generally assumed that inhibition of NF-κB alone will not dramatically affect most solid tumors, since they express other antiapoptotic factors. However, NF-κB inhibition may prove to be therapeutic in certain leukemias or lymphomas (such as Hodgkin's lymphoma), where NF-κB appears to play a unique survival role (Bargou *et al.*, 1997; Cabannes, 1999). Consistent with the hypothesis that NF-κB may be an important target for chemopreventive compounds, NF-κB is inhibited by aspirin or other nonsteroidal anti-inflammatory drug (NSAID) treatments (Yamamoto *et al.*, 2001), which block the initiation and/or progression of certain cancers. Interestingly, aspirin has been reported to inhibit the activation of the IκB kinase complex (Yamamoto *et al.*, 2001). Several dietary chemopreventive compounds, including flavonoids, curcumin, and resveratrol, are known to block NF-κB activation (Yamamoto *et al.*, 2001). These studies strongly support the hypothesis that NF-κB is a functionally relevant target of chemopreventive drugs and dietary compounds, possibly indicating the role of NF-κB is primarily in the earliest stages of oncogenesis.

3. Natural compounds as anti-inflammatory remedies

For centuries, natural compounds have been used as anti-inflammatory remedies, and considerable attention has focused recently on identifying the active components of these compounds. Several compounds have been purified, and

some have been shown to inhibit NF-κB at concentrations comparable to those of classical anti-inflammatory drugs (Nomura *et al.,* 2000). Green tea polyphenols and resveratrol, a polyphenol present in red wine, inhibit NF-κB activation in vitro by blocking the activity of IKK (Yamamoto *et al.,* 2001). Anti-inflammatory sesquiterpene lactones derived from various classes of medicinal plants also act, via inhibition of IκB phosphorylation, to block NF-κB activation in cell types ranging from T-cells and macrophages to fibrosarcoma and epithelial cells (Pan *et al.,* 200). Curcumin (i.e., a yellow pigment from turmeric) and capsaicin (i.e., a pungent component of red pepper that exhibits profound anticarcinogenic and antimutagenic activities) are potent inhibitors of IKK activity in several cell types (Surh, 2000). The application of curcumin onto the dorsal skin of mice significantly attenuates phorbol ester-induced NF-κB activation (Fisher *et al.,* 2001).

Glucosamine has received considerable attention in a number of studies over the past 5 years as an agent that may be beneficial for arthritis. Sold mainly over-the-counter in various formulations (glucosamine sulfate and glucosamine sulfate with chondroitin sulfate), researchers suggest that the beneficial effects of their compounds are due to the reconstruction of joint cartilage, one of the constituents of which is glucosamine in the form of glycoproteins of structural proteoglycans. A recent study in humans demonstrated some beneficial effects of glucosamine in arthritis (Reginster *et al.,* 2001) but the actual mechanism by which glucosamine may benefit the patient remains unknown. However, a recent report by Gouze et al. (2002) demonstrated glucosamine-dependent inhibition of NF-κB activity in rat chondrocytes and IL-1 bioactivity by up-regulation of the type II IL-1 decoy receptor. Also another very recent investigation suggests that it may interfere with pro-inflammatory cytokine action on human chondrocytes (Shikhman *et al.,* 2001). In this study, the ability of the newly synthesized quaternized glucosamine derivative to regulate NF-κB greatly contributed to its potency to inhibit MMP expression in HT1080 cells. This observation suggested testing its potential to act as an anti-inflammatory agent via inhibiting NF-κB pathway in macrophages.

Moreover, NF-κB is an important transcriptional factor that thought to act as a cellular apoptotic switch. Blocking the NF-κB pathway enhances the sensitivity of cells to apoptosis-inducing stimuli. Therefore, NF-κB inhibitory activity of QAGlc is favorable for its anticancer potential. In this context, its worth to study the NF-κB inhibitory potential in immune cells to elucidate its mechanism and to identify related effects resulted from inhibition of NF-κB activity in immune cells. Therefore, this part of the study was designed to study NF-κB inhibitory potential of QAGlc in immune cells.

Materials and Methods

1. Materials

Mouse macrophage tumor cell line, RAW264.7 was obtained from American Type of Culture Collection (Manassas, VA, USA). All the materials required for culturing of cells including cell culture media were purchased from Gibco BRL, Life Technologies (USA). MTT reagent, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin-streptomycin. For experiments, cells were detached with trypsin-EDTA and plated onto 24- or 96-well plates at a plating density of 7×10^5 and 1.5×10^5 per well, separately.

3. Cytotoxicity determination of RAW264.7 cells following treatment of QAGlc

To assess cell cytotoxicity levels of Glc and QAGlc on RAW264.7 cells, MTT assay was performed with different concentrations and incubation time intervals using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen *et al.* (1989). For this purpose, cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of Glc and QAGlc. After 48 h of incubation, cells were rewashed and 20 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to

the control (OD of treated cells - OD of blank / OD of control - OD of blank×100) and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $P<0.05$ was considered significant.

4. Reporter gene assay

RAW264.7 cells cultured in 10 cm^3 culture dishes were transiently transfected with NF-κB promoter containing luciferase reporter vector. Beta-galactosidase expression vector was co-transfected with the reporter vector to serve as an internal control of transfection efficiency. Transfected cells sub-cultured in 24-well plates were treated with different concentrations of QAGlc and Glc. Following stimulation with TNF-α (6 ng) and LPS (1 μg) cells were washed once with cold PBS and lysed with 200 μl/well lysis buffer (25 mM Tri-HCl, pH 8.0, containing 2 mM DDT and 1% Triton-X 100). Aliquots of cell lysate and luciferase substrate (Promega) were mixed in equal amounts in a 96-well plate and luminescence intensity was measured with a luminescence microplate reader (Tecan Austria GmbH, Austria). Beta-galactosidase activity was measured with ONPG buffer. The level of reporter gene expression was determined as a ratio, compared with cells stimulated by PMA (10 ng/ml) alone and represented as relative luciferase activity.

Transfection efficiency was determined by X-Gal staining method. Briefly, transfected cells were fixed with 0.5% glutaraldehyde and stained with X-Gal solution containing 20 mM $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and 1 mM $MgCl_2$. After 24 h of incubation at 37°C, transfected cells were visualized with blue color under a light microscope.

5. Extraction of nuclear and plasma protein

For separate extraction of nuclear and cytoplasmic proteins, CelLytic™ NuCLEAR™ Extraction kit (S26-36-23, Sigma-Aldrich Co., MO, USA) was used following manufacturer's instructions. Briefly, cells treated with QAGlc were collected and lysed with 0.5 ml of lysis buffer (500 μl, hypotonic lysis buffer, 5 μl,

0.1 M DTT, 5 μl protease inhibitor cocktail) and incubated for 15 min on ice. Igepal CA-630 solution (36 μl) was added and vortex for 20 seconds. Nuclei were separated by centrifugation at 13,000 × g and supernatant (cytoplasmic protein) was collected. Precipitated nuclei were lysed with 70 μl of extraction buffer mix (98 μl, extraction buffer, 1 μl of 0.1 M DTT and 1 μl protease inhibitor cocktail) for 30 min and nuclei protein were collected by centrifugation at 13,000 × g.

6. Western blot analysis

After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Briefly, harvested cells were washed twice with ice-cold PBS; resuspended in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) containing antipain (1 μg/ml), aprotinin (1 μg/ml), chymostatin (1 μg/ml), leupeptin (0.1 μg/ml), pepstatin (1 μg/ml), and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and incubated on ice for 20 min. Supernatants were recovered by a 10-min centrifugation (12,000 x *g*) at 4 °C, and protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin as a standard. Proteins (20-40 μg) were diluted in 5x sample buffer (10% SDS and 100 mM each dithiothreitol, glycerol, bromphenol blue, and Tris-HCl) and resolved in 4-20% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane,. Then proteins were transferred onto nitrocellulose membranes, and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with the appropriate dilution of primary antibodies (1:500) related to apoptosis and cell cycle progression. After three 5-min washes with Tris-buffered saline and 0.1% Tween 20, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. They were then washed again three times with Tris-buffered saline and 0.1% Tween 20, rinsed briefly with PBS, and developed with enhanced

chemiluminescence reagents (ECL, Amersham Biosciences, UK). Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). Detection of β-actin (1:5000 antibody dilutions) was used as control for equal loading of protein.

7. Determination IL-1β, IL-6 and TNF-α activity following treatment of QAGlc

Production of IL-1β, IL-6 and TNF-α in RAW264.7 cells was assayed using Biotrak™ ELISA kits (Amersham Pharmacia Biosciences, NJ, USA) following the instructions. Cells were treated with different concentrations of test material for 1 hand production of IL-1β and TNF-α was stimulated by 1 μg/ml final concentration of LPS and incubation was continued for another 24 h. Following incubation, conditioned medium was used for the experiment. For that, 50 μl of IL-1β and TNF-α standards (prepared for calibration) or same volume of test samples were added to wells of IL-1β or TNF-α antibody-coated 96-well plates in duplicate. Biotinylated antibody reagent (50 μl) was added and incubated for 3 h at room temperature. Reaction mixture was aspirated and washed using a microplate washer (Tecan Austria GmbH, Austria). Streptavidin-HRP conjugate (100 μl) was added and incubate for 30 min at room temperature. After complete washing, 100 μl of TMB substrate solution was added and incubated for 30 minutes at room temperature and reaction was stopped by adding 100 μl of stop solution. Optical density was determined at 450nm using GENios® microplate reader (Tecan Austria GmbH, Austria).

8. Determination PGE_2 activity following treatment of QAGlc

RAW 264.7 cells were cultured in 24-well plates with serum free media and pre-treated with different concentrations of QAGlc for 1 h. Production of PGE_2 was stimulated by adding 1 μg/ml final concentration of LPS and incubated for 24 h. The conditioned medium was used for PGE_2 determination by Biotrak™ Prostaglandin E_2 direct assay kit (Amersham Pharmacia Biosciences, NJ, USA)

according to manufacture's instructions. For this purpose 50 μl supernatant (includes PGE2 released from cells) from each treatment group was added into anti-mouse antibody coated wells and mixed with 50 μl of diluted mouse-anti PGE2. Plate was incubated at room temperature (20–25 °C) for 1 hour on a microplate shaker following addition of 50 μl of diluted conjugate into all wells except the blank. The reaction mixture was then aspirated and wells were washed with wash buffer. Immediately 150 μl of room temperature equilibrated TMB enzyme substrate was pipetted into all wells. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 minutes at room temperature (15–30 °C). The reaction was halted by the addition of 100 μl of 1 M Sulfuric acid to all wells. The optical density was read at 450 nm after 30 min using GENios® microplate reader (Tecan Austria GmbH, Austria).

9. Statistical analysis

Results are presented as mean ± standard error of the mean (n = 3). Student's t-test was used to determine the level of significance.

Results and Discussion

1. QAGlc inhibits NF-κB promoter activity

To investigate whether QAGlc could modulate the NF-κB promoter activity, RAW264.7, mouse macrophage cells were co-transfected with a NF-κB-promoter containing luciferase reporter construct and β-galactosidase expression vector. According to results of related studies, a wide variety of stimuli has been shown to induce NF-κB activity. Among them, TNF-α and LPS have been widely employed to stimulate NF-κB to identify NF-κB inhibitors. Therefore, transfected cells were then treated with nontoxic concentrations of QAGlc. To see the effect of QAGlc on NF-κB promoter activity, transfected cells were stimulated using bacterial endotoxin LPS and TNF-α. The results obtained from the reporter gene assay presented the potential of QAGlc to inhibit NF-κB promoter activity regardless of type of stimulation. TNF-α and LPS both stimulants resulted an almost similar induction of NF-κB promoter activity in transfected cells. A dose dependent decrease in luciferase activity that represents the suppression of NF-κB promoter activity was observed following treatment of QAGlc in both types of NF-κB stimulations (Fig. 1 and 2). Concurrent reductions in NF-κB promoter activities in glucosamine treated groups were observed, but these activities were much lower than that of QAGlc. Therefore, these results suggested the potency of this novel glucosamine derivative having quaternized amino functionality to affect transcriptional regulation of NF-κB.

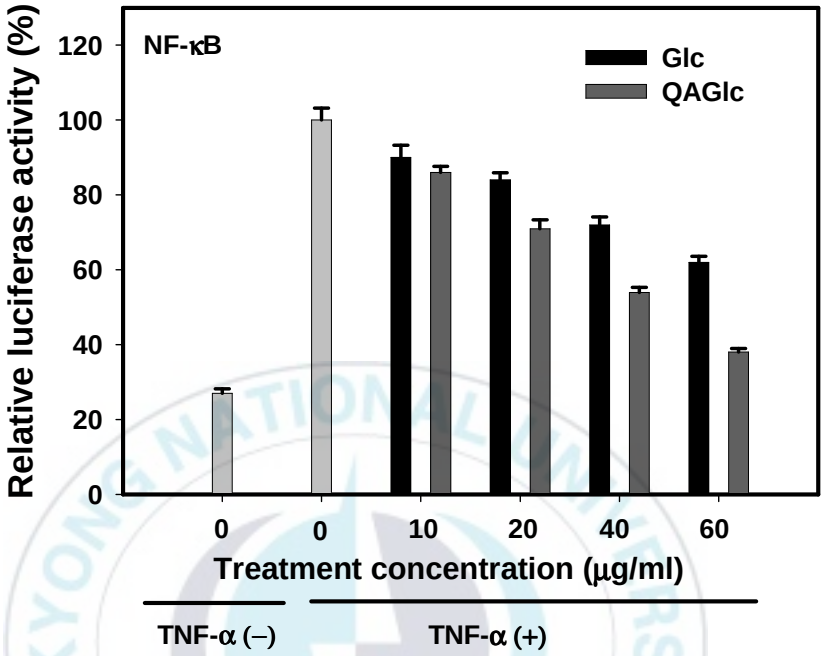

Fig.1. Suppression of NF-κB gene promoter activity in QAGlc treated RAW264.7 cells. Cells were co-transfected with NF-κB-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with TNF-α. Luciferase enzyme activity that represents NF-κB promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

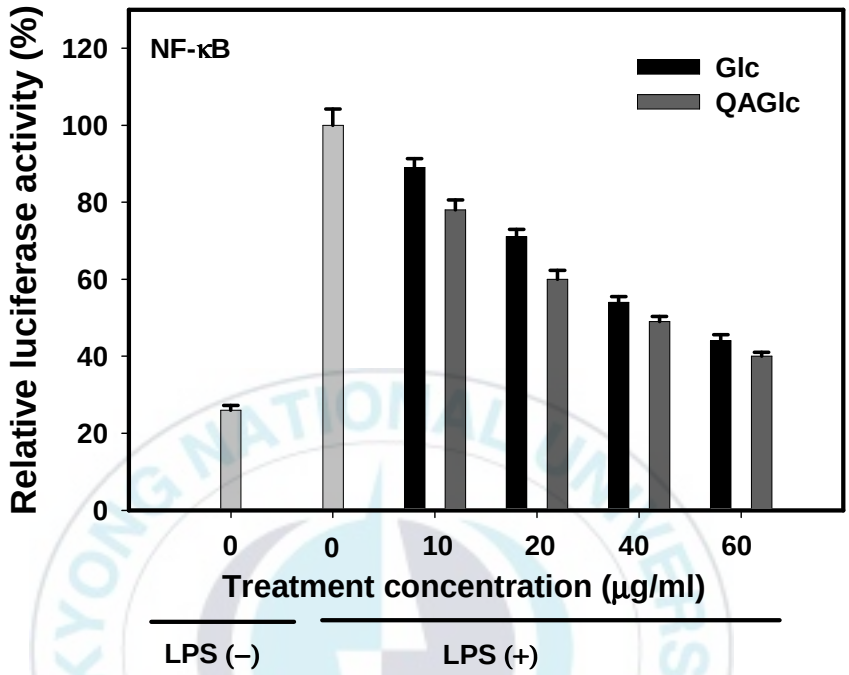

Fig.2. Suppression of NF-κB gene promoter activity in QAGlc treated RAW264.7 cells. Cells were co-transfected with NF-κB-luciferase reporter vector and β-galactosidase expression vector by lipofectamine. After 24 h of transfection, cells were subcultured into 24 well plates and treated with different concentrations of QAGlc followed by stimulation with LPS. Luciferase enzyme activity that represents NF-κB promoter activity was determined using luciferin as the substrate. The level of reporter gene expression was determined as a ratio, compared with non treated cells and represented as relative luciferase activity.

2. QAGlc reduces nuclear translocation of NF-κB

NF-κB has to be released from its inhibitory proteins, the IκBs in the cytosol, in order to translocate to the nucleus and activate target gene expression. To delineate the role of QAGlc on NF-κB translocation, plasma and nuclear protein extracts were separated from QAGlc treated LPS-stimulated cells. NF-κB protein expression was then tested using western blot analysis. Lower NF-κB levels were observed in plasma protein analysis of QAGlc treated cells compared to LPS treatment group. This reduction was much prominent in 40 and 60 µg/ ml concentrations of QAGlc. Moreover, this was much higher than that of aspirin at the similar concentration. Interestingly a clear reduction in translocation of NF-κB to nucleus in the presence of QAGlc was observed when nuclear extracts were analyzed. Therefore these results suggested the the involvement of QAGlc to mediate inhibition of NF-κB activation and translocation (Fig. 3).

Activation of NF-κB is preceded via induction of cascade of molecules. Therefore it was hypothesized that QAGlc could affect different targets of the NF-κB signaling cascade. Therefore, the level of NIK (NF-κB inducing kinase), IKK, IκB in QAGlc treated RAW264.7 cells were studied using western blot analysis. NIK has been reported to mediate the NF-κB activation triggered by LPS. The results revealed that treatment of QAGlc did not exert any effect on protein expression level of NIK (Fig. 4). However, treatment of QAGlc clearly inhibited protein expression levels of IKK and IκB in a concentration dependent manner (Fig. 4). These results revealed the primary target of QAGlc was IKK. This would be a logic step of QAGlc to target NF-κB inhibition because IKK is the critical kinase involved in activating the NF-κB pathway. Where, IKB is likely to play an accessory role. This type of compounds may be used for inhibiting IKK-ß phosphorylation of IκB which prevents subsequent degradation and activation of NF-κB dimers. Such methods are useful in the treatment of a variety of diseases associated with NF-κB activation mainly inflammatory diseases.

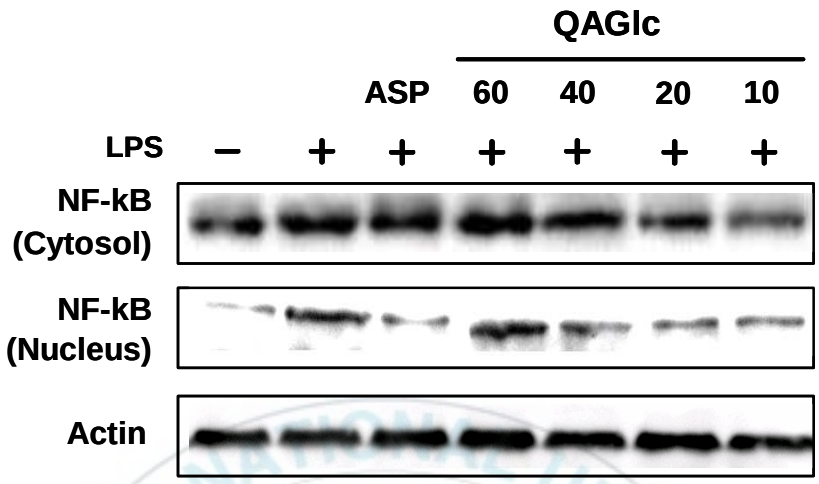

Fig. 3. Western blot analysis of NF-κB protein expressions in RAW264.7cells treated with QAGlc. After treatment of QAGlc, proteins in nucleus and cytosol were extracted and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. LPS: bacterial lipopolisaccharide, ASP: Aspirin.

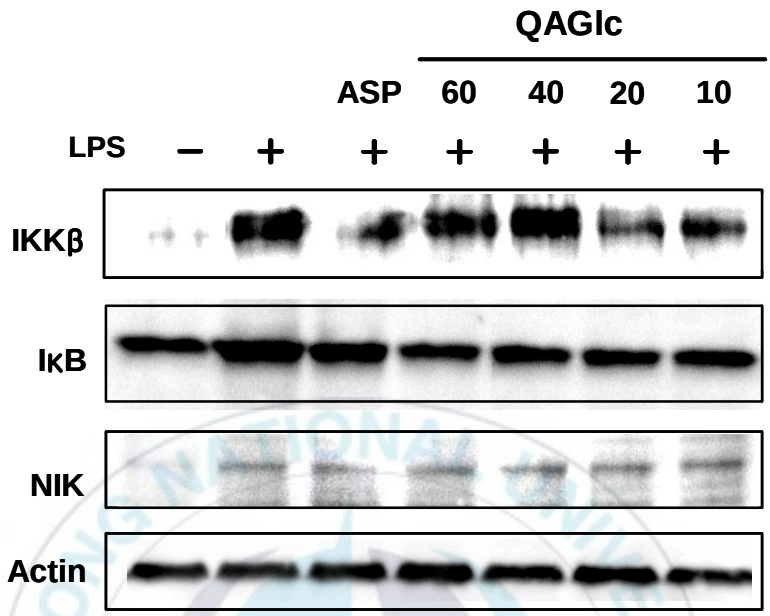

Fig. 4. Western blot analysis of IKKβ, IκB and NIK, protein expressions in RAW264.7 cells treated with QAGlc. After treatment of QAGlc, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. LPS: bacterial lipopolisaccharide, ASP: Aspirin

3. Effect of QAGlc on activities of inflammatory cytokines

Having confirmed that the QAGlc altered the translocation of NF-κB, the next set of experiments were sought to establish the alterations of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and PGE_2. For that, RAW264.7 cells treated with QAGlc were stimulated with LPS to assess the effect of QAGlc on the production of inflammatory mediators in NF-κB pathway. As expected, treatment of QAGlc suppressed the production of TNF-α (Fig. 5) IL-1β (Fig. 6), IL-6 (Fig. 7) and PGE_2 (Fig. 8) to different extents. These results were in agreement with the suggestion that the activation of NF-κB and the enhanced translocation of NF-κB lead to induction of NF-κB dependent cytokines including TNF-α, IL-1, IL-6, IL-8 and PGE2. Once these cytokines are activated they further increase the activity of NF-κB. In particularly, about 45% inhibition of LPS stimulated TNF-α activity was observed when QAGlc was treated with 50 µg/ml concentration. Suppression of TNF- α is a good feature of QAGlc as a material act against inflammation. Because TNF-α produced mainly by monocytes and macrophages exerts diverse effects in pathogenesis of number of chronic diseases by stimulating other cells and inducing tissue damage. Also it engaged in production of other types of inflammatory cytokines. Treatment of QAGlc at 50 µg/ml concentration suppressed the IL-1β concentration from 160 pg/ ml to 100 pg/ml. Much higher reduction was observed in IL-6 level when the cells were treated with QAGlc at the same concentration. It was about 3-fold decrement from the LPS group. Nearly 50% inhibition of PGE2 activity was observed when QAGLc treated to RAW264.7 cells at 50 µg/ml concentration. Further, protein levels of these inflammatory cytokines in the presence of QAGlc were assessed using western blot analysis. In line with the results obtained from immuno assays, a clear decrement in protein expression levels of TNF, IL-1 and IL-6 was observed (Fig. 9) when cells were treated with QAGlc compared to LPS treatment group alone.

4. Effect of QAGlc on protein expressions of ERK, JNK, and p38.

To elucidate whether QAGlc exert any effect on mitogen activated protein kinases (MAPK), their protein levels following treatment of QAGlc were assessed using western blot analysis. Treatment of LPS directly activates this pathway in macrophage cell lines. The results of this study clearly exhibited that treatment of QAGlc clearly inhibited mainly JNK and p38 MAPKs. However, a clear reduction of ERK could not be observed in RAW264.7 cells treated with different concentration of QAGlc (Fig 10). Production of cytokines has been reported to induce intracellular signaling transduction pathways including MAPK pathway. However, the results of this study are in agreement with the other reported findings. One related study has come up with the finding that blocking the activity of p38 prevents IL-1 and TNF-α production in LPS-stimulated monocytes at the level of translation (Lee *et al*., 1994). It has also been reported that dexamethasone, one of the well known steroidal anti-inflammatory drug inhibits TNF-α production at least in part by blocking induction of JNK (Swantek *et al*., 1997). Therefore, taken results of the present study together, it can be presumed that QAGlc inhibits NF-κB via combined alterations of MAPK and cytokines other than by regulating IKK.

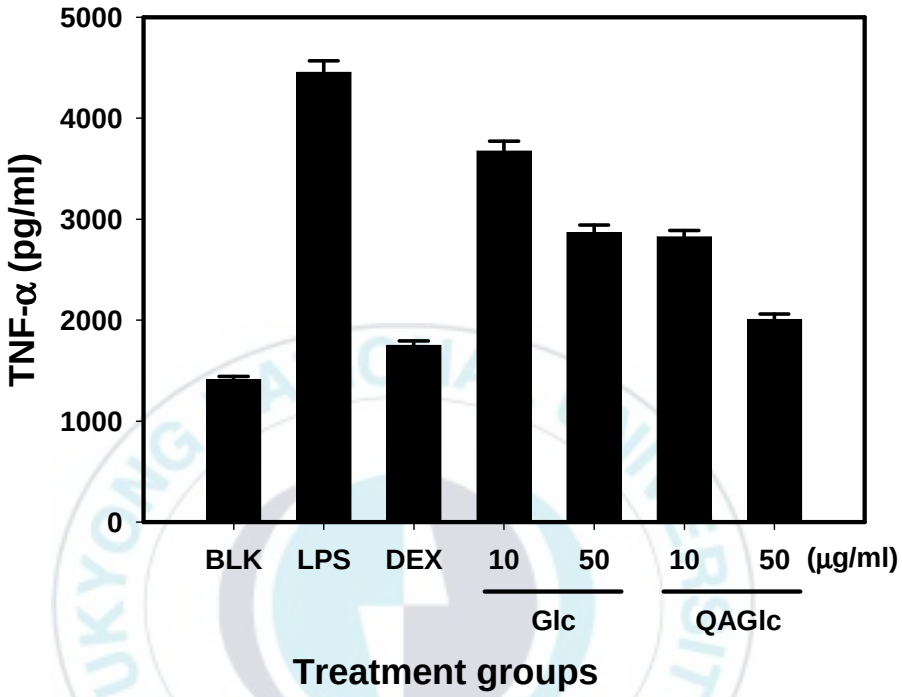

Fig. 5. Effect of QAGlc and Glc on TNF-α production in LPS stimulated RAW264.7 cells. Cells were treated with different concentrations of test materials or 1 μM dexamethasone (DEX) as a positive control for 1 h, and stimulated with LPS (1 μg/ml) for another 4 h. Following incubation, amount of TNF-α released was determined by TNF-α antibody coated ELISA kit as described in the materials and methods section.

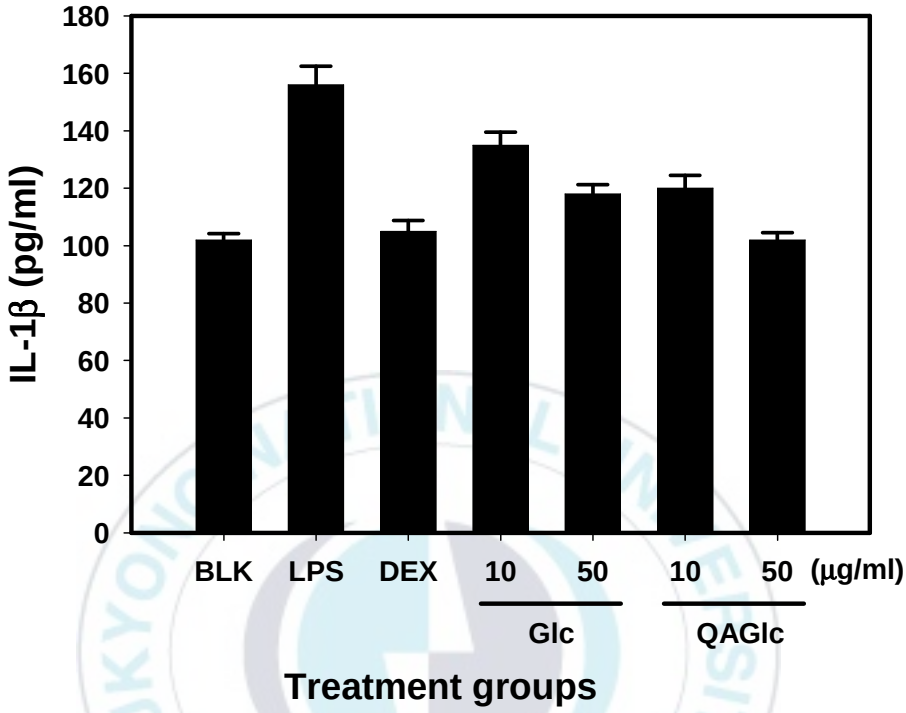

Fig. 6. Effect of QAGlc and Glc on IL-1β production in LPS stimulated RAW264.7 cells. Cells were treated with different concentrations of test materials or 1 μM dexamethasone (DEX) as a positive control for 1 h, and stimulated with LPS (1 μg/ml) for another 24 h. Following incubation, amount of IL-1β released was determined by IL-1β antibody coated ELISA kit as described in the materials and methods section.

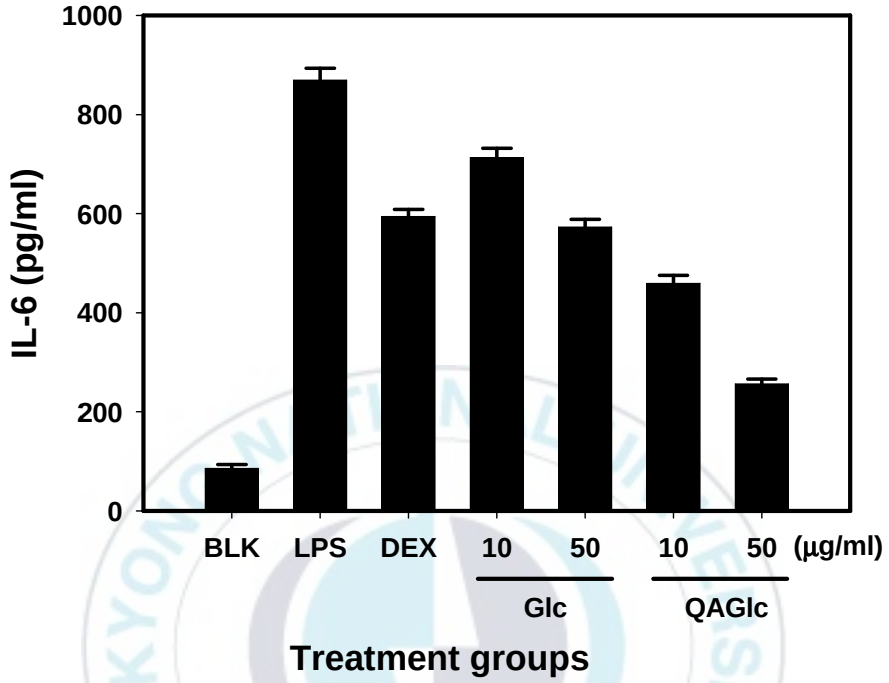

Fig. 7. Effect of QAGlc and Glc on IL-6 production in LPS stimulated RAW264.7 cells. Cells were treated with different concentrations of test materials or 1 μM dexamethasone (DEX) as a positive control for 1 h, and stimulated with LPS (1 μg/ml) for another 24 h. Following incubation, amount of IL-6 released was determined by IL-6 antibody coated ELISA kit as described in the materials and methods section.

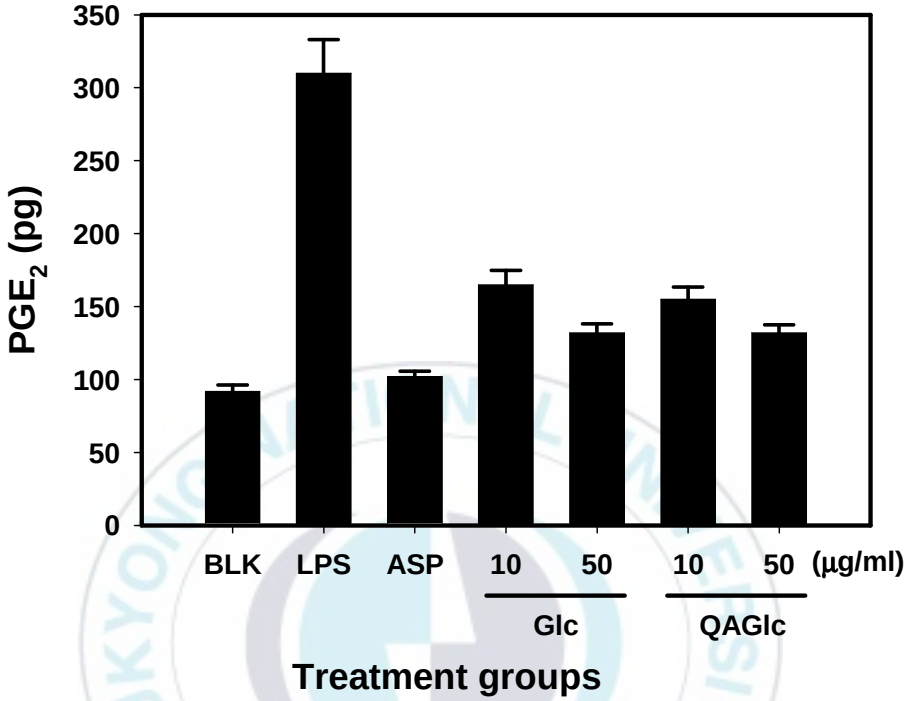

Fig. 8. Effect of QAGlc and Glc on PGE_2 production in LPS stimulated RAW264.7 cells. Cells were treated with different concentrations of test materials or 10 μM aspirin (ASP) as a positive control for 1 h, and stimulated with LPS (1 μg/ml) for another 4 h. Following incubation, amount of TNF-α released was determined by TNF-α antibody coated ELISA kit as described in the materials and methods section.

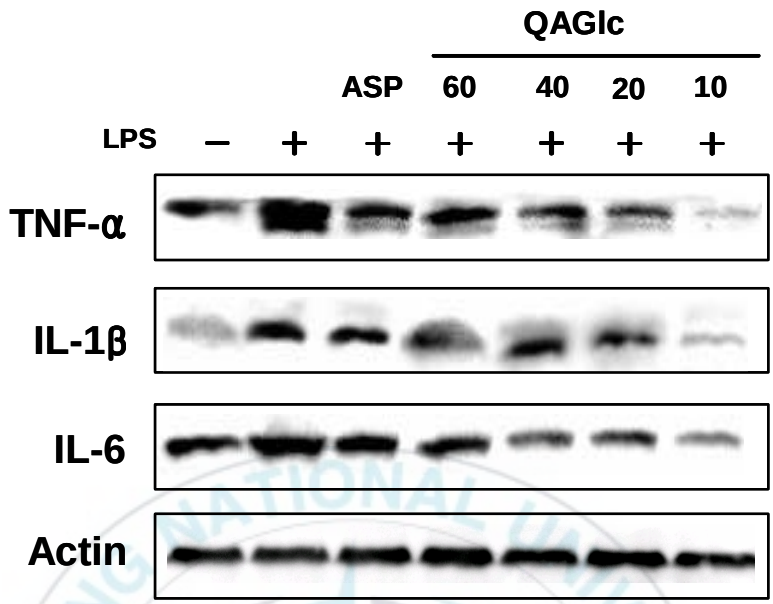

Fig. 9. Western blot analysis of TNF-α, IL-1β and IL-6 protein expressions in RAW264.7 cells treated with QAGlc. After treatment of QAGlc, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. LPS: bacterial lipopolisaccharide, ASP: Aspirin

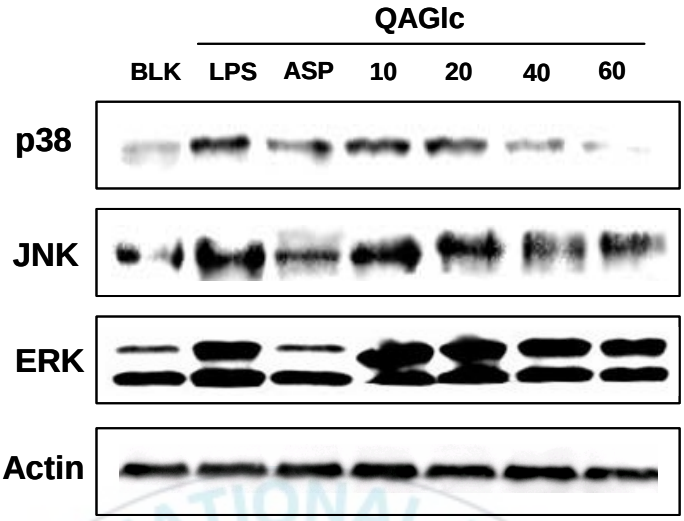

Fig. 10. Western blot analysis of p38, JNK and ERK protein expression in RAW264.7 cells treated with QAGlc. After treatment of QAGlc, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Proteins (20-40 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. LPS: bacterial lipopolisaccharide, ASP: Aspirin

5. Summary

Regulation of the NF-κB pathway is critical for the expression of genes involved in inflammation, immunity, and carcinogenesis. This study relates in general to a method of inhibiting pathological activation of the transcription factor NF-κB using novel glucosamine derivative substituted with quaternized amino functionality. The results of the present study revealed the NF-κB inhibitory potential of QAGlc. The QAGlc suppressed the promoter activity of NF-κB in transfected cells regardless of type of stimulation. Further, QAGlc altered the NF-κB activity and translocation in RAW264.7 cells. More specifically, it was revealed that QAGlc inhibits IKK, an important site for integrating signals that regulate the NF-κB pathway. Further, reduction in IKK led the subsequent reduction in IκB. Moreover, the amount and the activity of inflammatory cytokines, TNF-α, IL-1β, IL-6 and PGE2 were decreased in the presence of QAGlc confirming the link between these inflammatory mediators in the NF-κB signaling cascade. In agreement with the reduction in cytokines, a clear suppression of P-38 and JNK was observed when cells were treated with QAGlc. Therefore results of this study present a potent glucosamine derivative having an anti-inflammatory potential. This type of compounds is potential candidates for treating diseases in which activation of NF-κB is implicated.

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Anticancer Mechanism of a Novel Quaternized Amino Glucosamine derivative and Its Inhibitory Effects on Matrix Metalloproteinases and NF-κB

신규 합성된 Quaternized Amino Glucosamine 유도체의 항암기전 및 기질금속단백질분해효소와 NF-κB의 억제효능에 대한 연구

B. E. P. Mendis

Abstract

최근 연구에서는 몇몇 후보 항암제들의 활성을 결정하는데 분자들의 전하적 성질이 중요한 요소로 작용하는것으로 알려져 있다. 그러나 많은 연구에서 이러한 전하적 성질을 가지는 분자들(molecules)이 목표(target)가 되는 분자들 (molecules)과 작용기전이 서로 일치하지 않는다는 사실도 보고되고 있다. 본 연구에서는 Quaternized Amino (QA)가 분자량이 작은 강력한 전하적 성질을 나타내기 위해 글루코사민(glucosamine)으로 전환하였다. 이들 양이온을 띄는 Quaternized Amino Glucosamine (QAGlc)는 분자량이 적어서 흑색종세포에서 특이적인 독성효과를 나타내며, 이러한 사실은 기존연구에서 아직 밝혀지지 않는 새로운 사실이다. 그러므로 본 연구에서는 새로운 글루코사민(glucosamin) 유도체를 이용하여 흑색종 세포에 미치는 작용을 확인하였다. 즉 이들 분자들(molecules)의 농도와 처리시간에 따른 아포토시스(apoptosis) 현상을 측정하였다. 그 결과 QAGlc는 아포토시스(apoptosis) 신호전달과정에서 중요한 p53 유전자의 전사활성 및 단백질 발현을 증가시켰다. QAGlc는 p53 발현을 활성화시키데 관여하는 아포토시스 (apoptosis) 관련 단백질의 발현을 단계적으로 유도하였다. 뿐만 아니라 B16F1 세포에서 QAGlc은 Apaf-1 유전자 활성화을 통해 아포토시스(apoptosis)를 유발한다는 사실을 확인하였다. 또한 QAGlc은 HT1080 fibrosarcoma 세포에서 농도가 증가함에 따라 MMP-2 와 MMP-9 유전자의

전사가 억제된다는 사실을 알 수 있었다. 이들 MMP-2와 MMP-9는 AP-1과 NF-κB 전사인자를 감소시킴으로서 두 유전 자의 발현이 억제됨을 확인하였다. AP-1과 NF-κB 전사인자들은 mitogen-activated protein kinase (MAPKs)를 억제하는데 관여한다. 또한 면역세포들에서 QAGlc이 NF-κB을 강력하게 억제시켰으며, 이러한 사실은 RAW264.7 세포에서 QAGlc이 IKK 신호전달과정에 영향을 줌으로서 NF-κB가 억제된다는 것을 알 수 있었다.
결론적으로 QAGlc는 NF-κB 활성을 억제함으로서 MAPKs과 염증사이토카인 생성을 억제한다는 사실을 확인하였다.

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