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The inhibitory mechanism of a novel cationic glucosamine derivative against MMP-2 and MMP-9 expressions

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ABSTRACT

A number of recent researches have demonstrated the therapeutic effects of glucosamine (Glc) in a range of human diseases including arthritis. For the first time, we identified that a novel Glc derivative having quaternized amino functionality (QAGlc) suppresses MMP-9 and MMP-2, gelatinases in HT1080, human fibrosarcoma cells at 40 μg/ml, following stimulation with PMA. Reporter gene assay results revealed that, the mechanism of suppression involves decreased transcriptional activation of MMP-9 and MMP-2 via transcription factors NF-κB and AP-1. However based on western blot results, QAGlc did not attenuate the nuclear translocation of both NF-κB and AP-1. Apparently, phorbol myristate acetate (PMA) stimulated expressions of ERK, JNK and p38 were altered in the presence of potent tumour inducer, phorbol myristate acetate QAGlc, suggesting their suppression also contributes to QAGlc-mediated inhibition of MMP-9 and MMP-2. Moreover, the ability of QAGlc to inhibit gelatinases was confirmed by its ability to act against invasiveness of HT1080 cells through extracellular matrix components.

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all of the components of the extracellular matrix (ECM). 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, gelatinases, stromelysins, membrane type (MT)-MMPs, and non-classified MMPs.[1] MMP expression is increased and strongly correlated with tumor invasiveness and poor prognosis reinforcing the concept that MMPs contribute to human cancer development.[2] Among the MMPs, gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and possible use of their inhibitors has been suggested in the future to augment treatment strategies in specific cancers.

MMPs are highly regulated at the levels of both gene expression and protein activation. Transcriptional regulation of MMP genes is frequently suggested to be mediated by an AP-1 regulatory element in their proximal promoter regions.[3] 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.[4] Moreover, MMP-2 promoter has a number of potential cis-acting regulatory elements.[5] However, in recent years, NF-κB-dependent activation of MMP-2 has been reported.[6]

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.[7] 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.[8] Persistent activation of MAPK 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.[9]

The naturally occurring inhibitors of MMP activity (TIMPs) were the first compounds to be considered for clinical development. Among TIMPs, TIMP-1 forms preferential complexes with pro-MMP-9, whereas TIMP-2 and TIMP-4 exhibit high affinity for pro-MMP-2 exerting substrate specific effects.[10] However, the lack of effective methods of systemic gene delivery has limited the clinical utility of this approach. The rational chemical design of MMP inhibitors made possible the synthesis of compounds with specific inhibitory activities against the MMP subtypes that predominate in certain diseases, such as cancer and arthritis. Several effective MMP inhibitors have also been identified from natural sources. They are mainly plant origin such as resveratrol, theaflavins, catechins, curcumin and genistein.

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Glucosamine (Glc) salts constitute a new class of nutraceutical components due to their suggested beneficial roles in the human body.[11,12] 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. QAGlc, a novel Glc derivative was identified as a potent MMP-9 and MMP-2 inhibitor in HT1080, human fibrosarcoma cells in an effort to screen number of Glc derivatives having different functional groups. The results of this study present the alteration of transcription factors, AP-1 and NF-κB, and other genes accompany their activation in the presence of QAGlc.

Preparation of QAGlc was carried out according to Huang et al.[13] Resulting QAGlc was purified using a 100 Da molecular weight cut-off membrane (Spectrum Laboratories Inc. RanchonDominguez, CA) and a Dowex-50WX2 cation exchange resin (Dow Chemical Company (Michigan, USA) using modified phenol-sulfuric acid method.[14] The structure of purified QAGlc was determined by ^{1}H NMR, ^{13}C NMR spectroscopy (JNM-ECP-400 (400 MHz) spectrometer, JEOL, Japan), elemental (C, N, and H) analysis (Elementar Analysesysteme, Elementar Vario, EL, USA), and IR spectroscopy (Spectrum 2000 FT-IR spectrophotometer, Perkin-Elmer, USA). FT-IR (KBr, r, cm^{-1}): 3411 (OH), 2929, 2805 (CH), 1639 (CO), 1480 (Me), 1309 (CN), 1115, 1092, 1033 (pyranose) cm^{-1}; ^{1}H NMR (D_2O, 400 MHz, ppm): δ 5.4, 4.9 (1H, H-1$_\alpha$, H-1$_\beta$), 3.2, 2.9 (1H, H-2$_\alpha$, H-2$_\beta$), 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, ppm) δ 89.2, 92.4 (C-1$_\alpha$, C-1$_\beta$), 54.5, 56.1 (C-2$_\alpha$, C-2$_\beta$), 69.3 (C-3), 76.1 (C-4), 71.4, 72.3 (C-5$_\alpha$, C-5$_\beta$), 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).

Cells (HT1080) cultured in DMEM supplemented with 10% fetal bovine serum, were treated with different concentrations of QAGlc and non-toxic concentrations were used for the experiment. MMP-9 and MMP-2 activities in conditioned media following treatment with cells were assayed by gelatin zymography as described previously.[15] It was clearly observed that latent forms of MMP-9 (proMMP-9) and MMP-2 (proMMP-2) were converted into their active forms followed by treatment with phorbol myristate acetate (PMA), a potent tumor inducer (Fig. 1A). As depicted in Figure 1B, PMA (10 ng/ml) enhanced the MMP-9 and MMP-2 expressions by about 60% and 90%, respectively, compared to that of non-stimulated cells over the time. However, size and the intensity of lytic zones resulted due to gelatinolytic activities of MMP-9 and MMP-2 were greatly reduced in the presence of QAGlc and it clearly indicated that expression and activation of MMP-9 and MMP-2 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 a concentration of 60 μg/ml QAGlc showed approximately 50% inhibition of MMP-9 led gelatinolytic activity. This activity was much clear than the effect of doxycycline (DOX), 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. However, Glc, the starting material of QAGlc did not exhibit any inhibitory activity against MMP-9 and MMP-2 at all concentrations employed.

To assess direct inhibitory activity against enzymes, conditioned media were treated with similar concentrations of doxycycline (DOX) and QAGlc and the reaction mixture was then zymographed according to the same above procedure.[15] In that experiment alterations in enzymatic activities of MMP-9 or

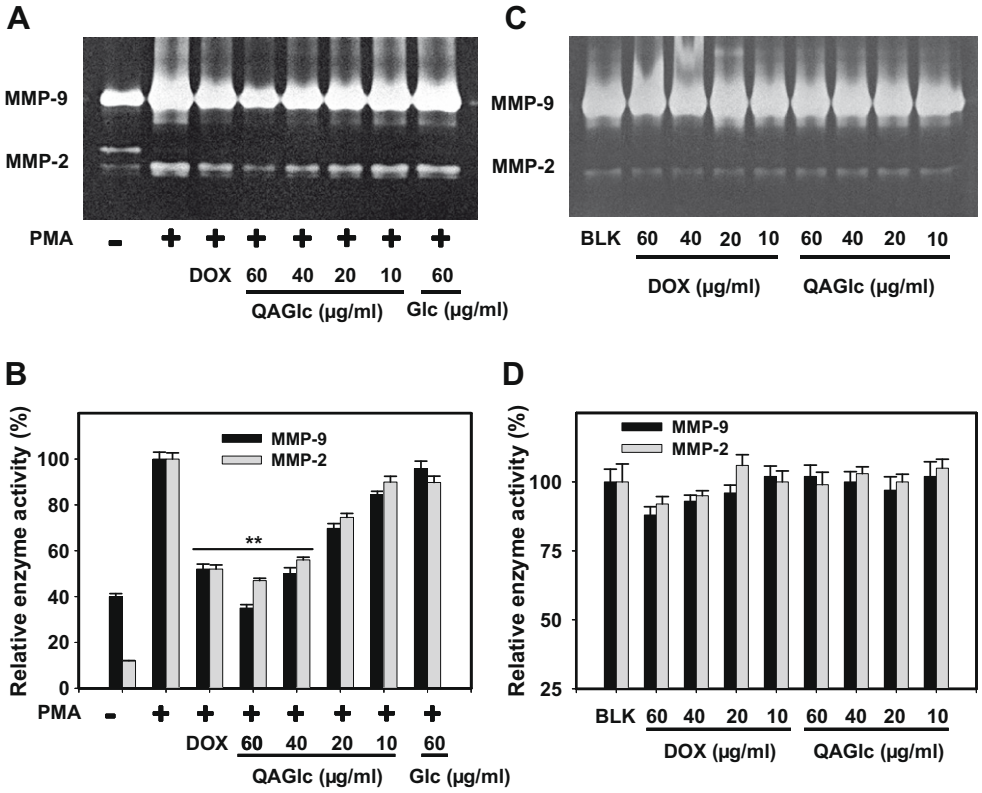

Figure 1. (A) MMP-9 and MMP-2 gelatinolytic activities in conditioned media of PMA stimulated HT1080 cells following treatment with QAGlc. The conditioned media was subjected to electrophoresis in a 10% (w/v) polyacrylamide gel impregnated with gelatin. Zymograms were developed in the presence of developing buffer, stained and the image of the gel was recorded using an image reader. Both MMP-9 and MMP-2 activities were significantly ($P < 0.01$) reduced by the positive control, doxycycline (DOX) and QAGlc at 40 and 60 μg/ml. (B) MMP-9 and MMP-2 activities as percentages in conditioned media of PMA stimulated HT1080 cells following treatment with QAGlc. (C) Direct inhibition of MMP-9 and MMP-2 gelatinolytic activities in conditioned media treated with QAGlc. Cells were stimulated with PMA and the conditioned media was treated with QAGlc. (D) MMP-9 and MMP-2 activities as percentages in conditioned media treated with QAGlc.

MMP-2 were not observed (Fig. 1C and D). However, in the presence of similar concentrations of DOX, slight decrements in enzymatic activities were observed at higher concentrations used.

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. A high (about four-fold) luciferase activity was noted in MMP-9 and MMP-2 transfected cells when stimulated with PMA (Fig. 2A). However, the luminescence reading values obtained for MMP-2 were greatly lower than that of MMP-9. These results correlated well 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 three-fold when the cells were treated with QAGlc at 60 μg/ml. Moreover, both MMP-9 and MMP-2 inhibitions followed a dose-dependant pattern.

When the cells were transfected with NF-κB promoter containing luciferase reporter vector, luciferase activity in PMA stimulated cells was increased by approximately three-fold whereas about 65% inhibition was observed once the cells were treated with QAGlc at 60 μg/ml (Fig. 2B). 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 luciferase vector followed by treatment with QAGlc.

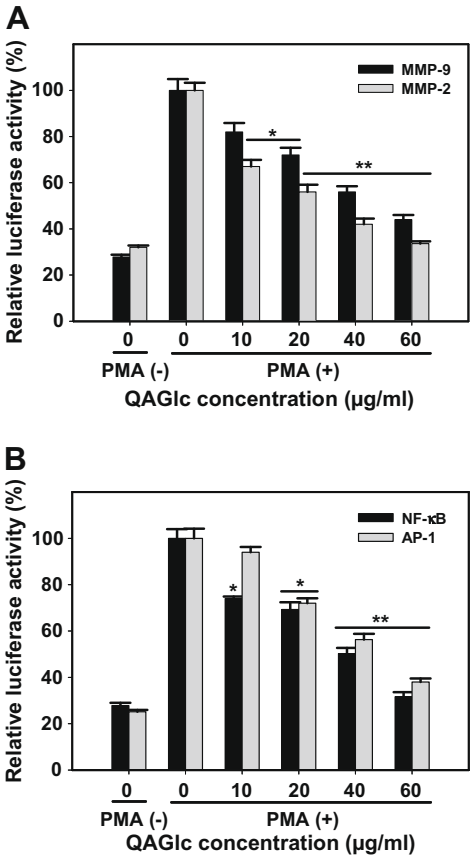

Figure 2. Suppression of gene promoter activities of (A) MMP-9 and MMP-2 and (B) NF-κB and AP-1 in QAGlc treated HT1080 cells. Separate experiments were carried out to co-transfect cells with luciferase reporter vectors and β-galactosidase expression vector by lipofectamine. Luciferase enzyme activities that represents promoter activities of relevant genes were 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. * = $P < 0.05$ and ** = $P < 0.01$ were considered statistically significant.

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.

To determine any alteration in protein expression levels of the signaling pathway following treatment with QAGlc, western blot analysis was performed with MMP-9 and MMP-2. A clear concentration dependent suppression effect was observed in MMP-2 and MMP-9 protein levels analyzed by western blot in the presence of QAGlc compared to that of the cells treated with PMA alone (Fig. 3A). 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. Further, to better understand whether down-regulation of MMP-9 gene expression was due to AP-1 transcriptional down-regulation or blockade of its translocation, c-Fos (a subunit of AP-1) protein levels in cytoplasm and nucleus were studied using western blot analysis. The results clearly showed that NF-κB (p65) and c-Fos (a subunit of AP-1) protein expression levels in both cytosol and nucleus repressed in the presence QAGlc following a similar trend (Fig. 3B and 3C). In contrast, significant difference ($P > 0.05$) in TIMP-1 and TIMP-2 protein levels was not observed in the presence of QAGlc even at relatively higher concentrations (Fig. 3D). Further, separate set of western blot experiments were performed to examine the ability of QAGlc to alter MAPKs that correlates well with MMP expression. As shown in Figure 4A QAGlc exerted inhibitory effects on main MAP kinases including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 that stimulated with PMA. More than 60% protein expression inhibition of ERK was observed when the cells were treated with QAGlc at 60 μg/ml. The effect of QAGlc towards protein expression of JNK and p38 was lower than that of ERK.

Further, the invasiveness of HT1080 cells through the ECM was assessed using a CHEMICON® Cell Invasion Assay Kit (ECM550, Chemicon International Inc., CA, USA). As shown in Figure 4B, after 36 h of incubation with PMA, a significant ($P < 0.05$) increment in invasion of HT1080 cells through the ECM layer was observed. In contrast, invasiveness of cells was clearly inhibited in the presence of QAGlc. However, inhibition of invasiveness observed in the presence of Glc was not significant ($P > 0.05$) compared to that of QAGlc. In the presence of 20 μg/ml QAGlc, about 50% of the invasion was decreased after 48 h. The migration of HT1080 cells were blocked by 70% without affecting the cell viability when QAGlc concentration was increased up to 60 μg/ml.

The results presented herein confirmed that QAGlc, a novel cationic Glc derivative is an efficacious agent that can inhibit the expression and activation of MMP-9 and MMP-2 in HT1080 human fibrosarcoma cells that express high amounts of MMP-9 and MMP-2. This compound significantly ($P < 0.05$) decreased the latent as well as active forms of MMP-9 and MMP-2 stimulated with PMA interfering with the proteolytic activation of pro-MMP into their active forms. The inhibitory potency of QAGlc on MMP-9 and MMP-2 is highly unlike due to its toxicity since cell viability was not affected even with the highest concentration of QAGlc. Further, the potency of QAGlc to inhibit gelatinases was clearly observed with its ability to act against invasiveness of HT1080 cells stimulated with PMA. Because, during metastasis invading cells use these enzymes to degrade extracellular matrix and to invade tissues. Invasion and mobility assay used in this study, was an ideal method to assess the above effect of MMPs. Moreover, QAGlc interfered with direct transcriptional activation of MMP-9 and MMP 2 promoter activities and western blot analysis data confirmed a clear reduction in protein expression levels of MMP-9 and MMP-2 in the presence of QAGlc. On the contrary, comparative zymography results obtained with conditioned media treated with QAGlc

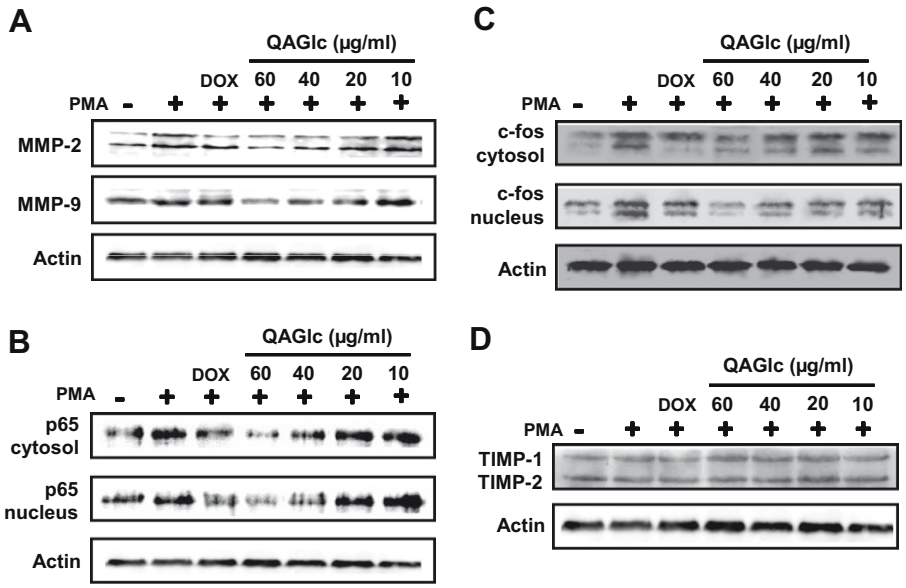

Figure 3. Western blot analysis of protein expressions in HT1080 cells treated with QAGlc. (A) MMP-2 and MMP-9 (B) NF-κB (p65) in cytosol and nucleus (C) c-fos in cytosol and nucleus (D) TIMP-1 and TIMP-2. After treatment of QAGlc cells were stimulated with PMA. Proteins present in nucleus and cytosol were then extracted and resolved by denaturing SDS–PAGE 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 luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein.

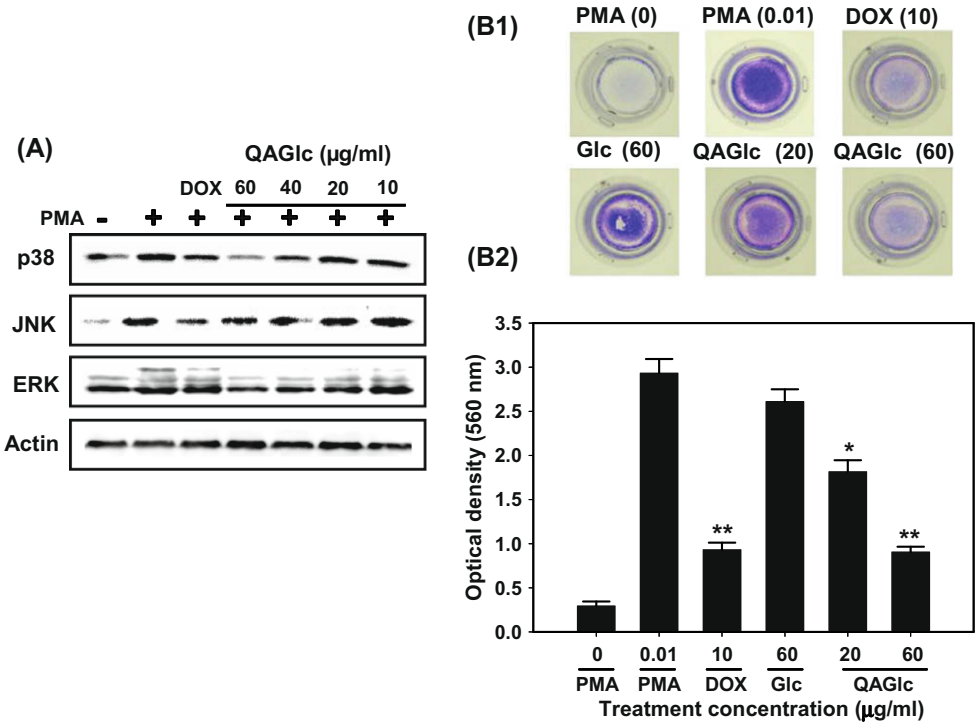

Figure 4. (A) Western blot analysis of ERK, JNK and p38 protein expressions in HT1080 cells treated with QAGlc. After treatment of QAGlc cells were stimulated with PMA and lyzed with lysis buffer. Proteins were then extracted and resolved by denaturing SDS–PAGE in 4–20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies, respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using luminescent image analyzer. Detection of β-actin was used as control for equal loading of protein. (B1) Cell invasion assay in HT1080 cells. Cell invasion through ECM containing basement membrane was analyzed by staining the cells that invaded the lower surface of the membrane. Stained cells that invaded through the membrane in different invasion chambers conducted with different treatment groups. (B2) Optical density resulted from the stained cells and expresses the amount of cells invaded. $^{*} = P < 0.05$ and $^{**} = P < 0.01$ were considered statistically significant.

or DOX, a tetracycline derivative that has reported to be involved in MMP inhibition via a number of mechanisms confirmed that QAGlc does not have direct involvement in inhibiting. MMP enzyme activities.

Interference with PMA stimulated AP-1 and NF-κB signaling by QAGlc is one possible explanation for the reductions in MMP-9 and MMP-2 expressions. Reporter gene assay results performed with expression vectors containing promoters of NF-κB and AP-1

transcription factors elucidated that QAGlc obstruct their transcriptional activation. 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.[16] On the basis of these collective findings, one could speculate, intriguing possibility that NF-κB and AP-1 may modulate the activity of each other, thus expanding the scope of QAGlc mediated down regulation of these transcription factors. Therefore, the inhibitory effects of QAGlc on MMP-9 and MMP-2 can be speculated to be regulated mainly via suppression of both NF-κB and AP-1 transcription factors. This agress with the findings of previous studies showing that NF-κB and AP-1 sites are important for both basal and PMA-induced transcription of human MMP-9.[17] Moreover, western blot analysis data confirmed QAGlc was involved in suppression of NF-κB protein expression. However, the levels of decrements in cytoplasm and nucleus were comparable arguing that it does not involve NF-κB nuclear translocation resulting in reduced levels of transcripts. Similarly, reduction in protein expression levels of cytoplasmic and nuclear c-Fos (a subunit of AP-1) was in consistence with AP-1 transcriptional down-regulation but not with its nuclear translocation.

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.[18] Moreover, the western blot analysis performed with monoclonal antibodies of phosphorylated forms of ERK, JNK and p38 clearly exhibited that QAGlc is capable of attenuating their protein expressions down to different extents. Therefore it can be presumed that inhibition of MAPK contributes to the inhibition of MMP-9 and MMP-2 expression via suppression of NF-κB and AP-1. However, the suppression was clearer with regard to ERK and it is much important in the context of MMP inhibition. In metastatic cells specifically ERK activity was shown to be higher when compared to non-metastatic cancer cells.[19] 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 AP-1.

Studies using small molecule inhibitors of MMPs in early disease provide evidence that MMP inhibitors would be useful as therapies to treat and prevent metastasis.[9] This study presents the first evidence of a cationic Glc derivative to be an efficacious agent that can attenuate expression and activation of MMP 9 and MMP-2 via mechanisms involving transcriptional regulation of NF-κB and AP-1 and related signaling pathways. The widespread availability of Glc and facile way of modifying its structure will very likely to yield a rational basis for the development of potential drug candidates in the treatment of disorders in which the MMP system becomes amplified, such as degenerative osteoarthritis, periodontitis, and cancer.

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