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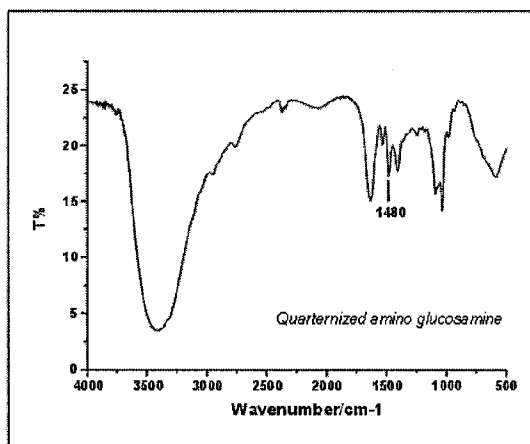
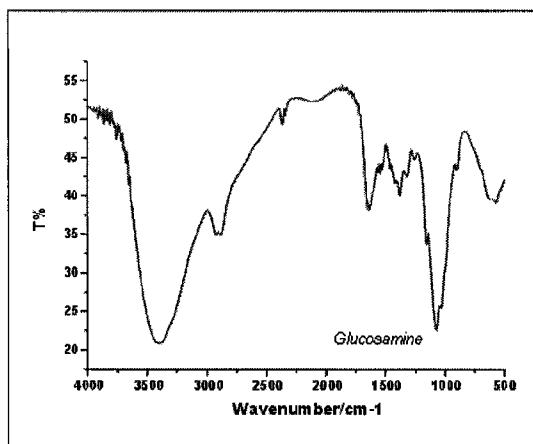
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(54) Title: QUARTERNIZED AMINO GLUCOSAMINE COMPOUND FOR EXERTING ANTICANCER EFFECT



(57) Abstract: The present invention relates to the quarternized amino glucosamine, it can exert an anticancer effect via induction of apoptosis in a dose and time dependant manner through which an anticancer treatment effect can be increased.

【DESCRIPTION】

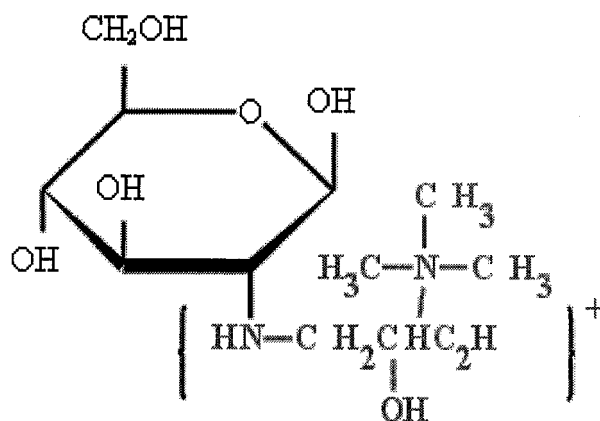
【Invention Title】

QUARTERNIZED AMINO GLUCOSAMINE COMPOUND FOR EXERTING ANTICANCER EFFECT

【Technical Field】

<1> The present invention relates to a quarternized amino glucosamine(QAGlc) compound of formula 1 having anticancer effect, more specifically, to the quarternized amino glucosamine compound which can exert an anticancer effect via induction of apoptosis in a dose and time dependant manner through which an anticancer treatment effect can be increased.

<2> <Formula 1>



<3>

【Background Art】

<4> Apoptosis is the mechanism of cell death activated in mammalian cells following exposure to a wide variety of stimuli including anticancer agents. The chemotherapeutic agents that have been identified as being apoptosis-inducing include etoposide, dexamethasone, vincristine, cis-platinum, cyclophosphamide, paclitaxel, 5'-fluorodeoxyuridine, 5'-fluorouracil and adriamycin. The efficiency of the anticancer agents seems to be related to the intrinsic propensity of the tumor cells to respond to these agents by apoptosis. This indicates that apoptosis may be the primary mechanism in antineoplastic agents.

<5> Apoptosis is an orchestrated series of events through which the cell precipitates its own death. The stages of apoptosis include cell shrinkage,

chromatin condensation, nuclear segmentation and internucleosomal fragmentation of DNA, resulting in the generation of apoptotic bodies. Induction of apoptosis has been used as an index to screen for new anticancer substances and to study the anticancer mechanisms of chemotherapeutic drugs.

<6> Glucosamine resulting from the hydrolysis of chitosan has been reported to exert several biological activities and recently there was a tendency to synthesize glucosamine derivatives having different functional groups important for some specific activities.

<7> The object of the present invention is to prepare quarternized amino glucosamine and to provide a composition for anticancer capable of increasing anticancer treatment effect by exerting anticancer effect via induction of apoptosis by quarternized amino glucosamine.

【Disclosure】

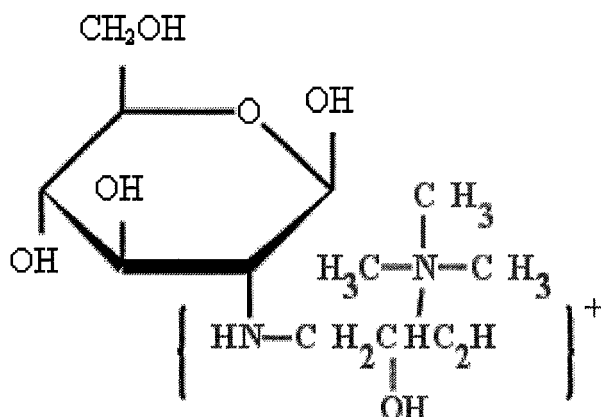
【Technical Problem】

<8> Such object of the present invention was accomplished by the method that glucosamine was prepared by hydrolyzing chitosan and quarternized amino glucosamine was obtained using glucosamine.

【Technical Solution】

<9> The present invention provides the quarternized amino glucosamine(QAGlc) compound having anticancer effect of formula 1.

<10> <Formula 1>



<11>

<12> The present invention also relates to a composition for anticancer

comprising the quarternized amino glucosamine of formula 1 as effective component.

<13> The composition for anticancer can be prepared in the form of inhalation powders, tablet, capsule, powders, ointment composition, solution, gel, paste, patch and granule.

<14> In the followings, the present invention will be illustrated with reference to examples and experimental examples. However, the present invention is not restricted by such examples.

【Advantageous Effects】

<15> The quarternized amino glucosamine of the present invention can exert an anticancer effect via induction of apoptosis in a dose and time dependant manner through which an anticancer treatment effect can be increased.

【Description of Drawings】

<16> FIG.1 is a graph showing FT-IR spectra of Glc and QAGlc.

<17> FIG.2 is a table showing cell cytotoxicity of QAGlc on various cancer cells with concentrations and time intervals dependently.

<18> FIG.3 is an image showing time dependant DNA damages induced by QAGlc in B16F1 cells.

<19> FIG.4 is an image showing concentration dependent DNA damage induced by QAGlc in B16F1 cells.

<20> FIG.5 is an image showing expression of p53 gene in B16F1 cells treated with QAGlc.

<21> FIG.6 is a graph showing induction of p53 gene expression assessed using reporter gene assay.

<22> FIG.7 is a graph showing induction of p21 gene expression assessed using reporter gene assay.

<23> FIG. 8 is an Western blot analysis of phospho p53 protein expression in B16F1cells treated with QAGlc.

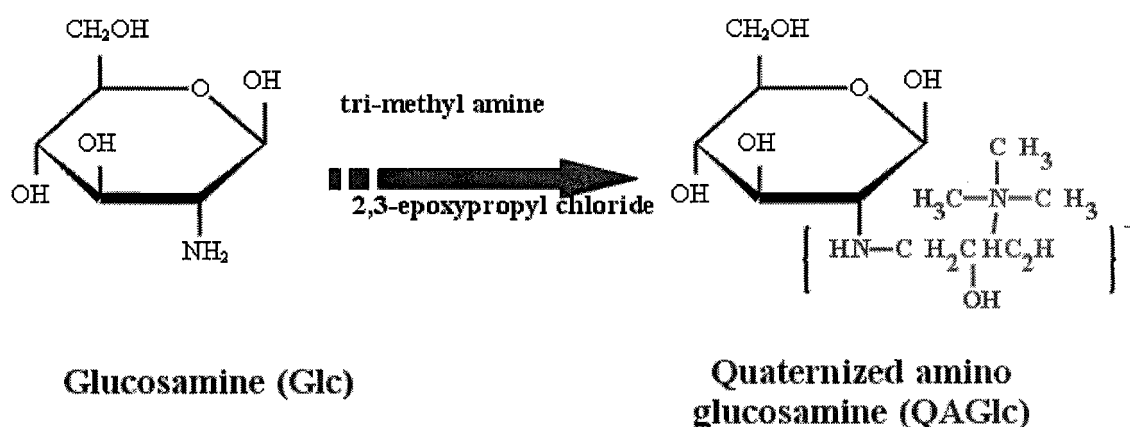
【Best Mode】

<24> Example 1: *Preparation of quarternized amino glucosamine*

<25> As shown in reaction formula 1, glucosamine was prepared from acidic

hydrolysis of chitosan by hydrolyzing chitosan with concentrated HCL for 3 h and resultant glucosamine hydrochloride was precipitated with 100% ethanol. Glucosamine hydrochloride was quarternized by reacting with 2,3-epoxypropyl chloride and tri-methyl amine.

<26> <Reaction formula 1>



<27>

<28> For the synthesis, pH of the trimethylamine solution was first adjusted to 2.0 to increase the boiling point. Then epoxypropyl chloride at same molar ratio was added dropwise and the pH was maintained at 9 using 1M NaOH solution. Glucosamine was then added at same molar ratio and reaction was proceeded by stirring for 24 h. Throughout the reaction pH was maintained at 9.0 product was extracted using ethanol, methanol and tri hydrofluoride and quaternized amino glucosamine was obtained as a dark brown fluffy solid. Structure of newly synthesis was confirmed by elemental analyses and FT-IR. The result of elemental analysis was demonstrated in table 1 and the result of FT-IR was shown in figure 1. Bend absorption observed at 1480 cm^{-1} in QAGlc by FT-IR, clearly confirmed the quarternization of glucosamine

(substitution of N (CH₃)₃ group).

<29> <Table 1>

	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

<30>

【Mode for Invention】

<31> **Experimental example 1: Anticancer effect**

<32> In order to assay the cell cytotoxicity of glucosamine and quarternized amino glucosamine on colon cancer cell(SW480), lung cancer cell(A549), mouse melanoma cell(B16F1), leukemia cancer cell(HL60) and liver cancer cell(Hep3B), MTT assay was performed with diffent sample concentrations and incubation time intervals

<33> Cells were cultured in microtiter 96-well plates with serum free media and treated with different sample concentrations for varying time intervals and anticancer effect was assessed using MTT cell viability assay. The result was represented in figure 2. QAGlc exerted a clear anticancer effect after 36 h of treatment. Moreover, the effect was concentration dependent.

<34> **Experimental example 2: *Detection of apoptosis by DNA fragmentation assay***

<35> In order to examine anticancer effect and mechanism of QAGlc, B16F1 cells cultured in 10cm culture dishes were pre-treated with different concentrations of QAGlc and apoptosis was induced with 2mM H₂O₂. Then, cell incubation was performed for different time periods. Cell was rinsed and total DNA was extracted with the standard procedure. DNA was electrophorased on a 2% agarose gel and DNA fraction was visualized using ethedum bromide staining. The result was represented in figure 3.

<36> DNA damage was first observed after 36 h with 100 $\mu\text{g/ml}$ concentration. Prolongation of incubation (after 36 h) exerted a clear effect DNA damage by all the tested concentrations dose-dependently.

<37> B16F1 cells cultured in 10cm culture dishes were pretreated with different concentrations of QAGlc and apoptosis was induced with 2mM H_2O_2 . After incubating for 48 h, genomic DNA was extracted and electrophorased on a 2% agarose gel. The result was represented in figure 4.

<38> QAGlc exerted a higher DNA damage dose-dependently, which shows induction of apoptosis. At a concentration of 100 $\mu\text{g/ml}$, QAGlc exerted a higher DNA damage which means instructed.

<39> **Experimental example 3: *Reverse transcription-polymerase chain reaction (RT-PCR)***

<40> B16F1 cells were treated with QAGlc and total RNA was extracted. PCR was carried out using RT-generated cDNA encoding p53, p21 and G3PDH with their specific primers to amplify p53 and G3PDH mRNA. PCR products were analyzed on a 2% agarose gel. The mRNA of G3PDH served as an internal control for sample loading and mRNA integrity. The result was represented in figure 5.

<41> After 24 h and 36 h of incubation, p53 transcription was up-regulated by QAGlc. This effect was much higher than that of etoposide, a well-known anticancer compound.

<42> **Experimental example 4: *Transfection and gene reporter assay***

<43> B16F1 cells were transfected with p53-luc, p21-Luc reporter vectors and β -galactosidase expression vector by lipofectamine (Invitrogen) transfection method. After 24 h of transfection, cells were subcultured into 24 well plates and cells treated with different concentrations of QAGlc. The luciferase activity value was normalized to transfection efficiency monitored by the cotransfected β -galactosidase expression vector. The level of induction of luciferase was determined as a ratio in comparison to cells with no stimulation. The result was represented in figure 6.

<44> p53 gene expression was up-regulated dose dependently by QAGlc and at

100 $\mu\text{g/ml}$ the effect was 2-fold than that of etaposide.

<45> According to the method described above, p21 gene was transfected to B16F1 cells according to the method described above and gene expression after the treatment of QAGlc was determined by relative luciferase activity. The result was represented in figure 7.

<46> All the concentrations of QAGlc significantly enhanced p21 gene expression compared to the control.

<47> **Experimental example 5: *Western blot analysis***

<48> After treatment of QAGlc for different time intervals, cells were lysed in RIPA buffer [10 mmol Tris-HCL (pH 7.4), 1% deoxycholate, 1% NP40, 150 mmol NaCl, 0.1% SDS, 0.2 mmol phenylmethyl sulfonyl fluoride, 1 mg/L aprotinin and 1 mg/L leupeptin] for 30 min on ice. The lysates were centrifuged at 12,000xg for 15 min to remove debris. Protein samples were resolved using a 420% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane, and blocked with 10% skim milk. Monoclonal antibodies of phosphorylated p-53 (Cat. No, Chemicon, CA, USA) were used to detect phosphor-p53 protein using chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. Western blot bands were quantified using ImageMaster software (Amersham Pharmacia Biosciences, NJ, USA). The result was represented in figure 8.

<49> As shown in figure 8, the results clearly exhibited that treatment of QAGlc increase phospho p53 protein level in a time dependant manner indicative of up-regulation of p53 gene expression.

<50> Glucosamine prepared from acidic hydrolysis of chitosan was quarternized by reacting with 2,3-epoxypropyl chloride and tri-methyl amine. FT-IR data clearly confirmed the quarternization of glucosamine with a bend absorption of $\text{N}(\text{CH}_3)_3$ at 1480 cm^{-1} .

<51> We could provide evidence that quarternized amino glucosamine exerts an anticancer effect on murinemelanoma cells (B16F1) via induction of apoptosis

in a dose and time dependant manner.

<52> B16F1 cells were treated with different concentrations of quarternized amino glucosamine and cytotoxicity was determined using MTT assay. A clear anticancer effect was observed after 48 h of incubation with concentrations below 0.01%. Apoptosis was confirmed by agarose gel electrophoresis of DNA fragmentation and FACS analysis clearly demonstrated that quarternized amino glucosamine increase the production of apoptotic cells. RT-PCR products exhibited that QAGlc induce apoptosis via induction of p53 and p21 genes in time dependant manner. This effect could be confirmed when the B16F1 cells were transfected with promoter plasmids of p53 and p21 treated with different concentrations of QAGlc. Induction of phosphor p53 observed in western blot analysis further confirmed that quarternized amino glucosamine induce apoptosis via p53 dependant pathway.

【Industrial Applicability】

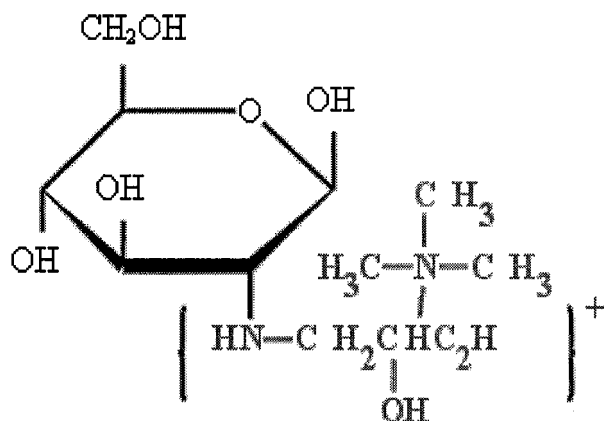
<53> The quarternized amino glucosamine of the present invention exerts an anticancer effect via induction of apoptosis in a dose and time dependant manner through which an anticancer treatment effect can be increased such that the present invention is medicalpharmaceutically useful invention.

【CLAIMS】

【Claim 1】

<55> A quarternized amino glucosamine of formula 1 having anticancer effect.

<56> <Formula 1>



<57>

【Claim 2】

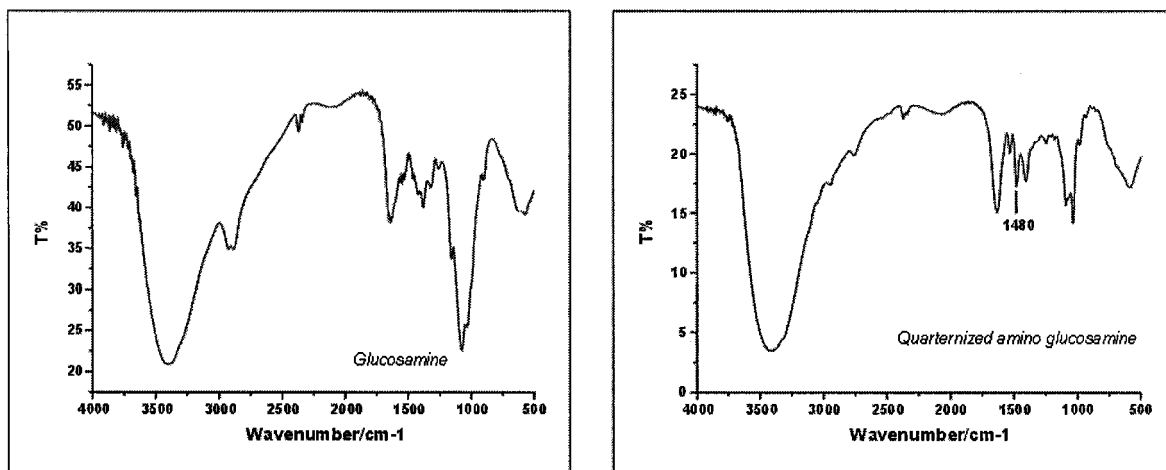
<58> A composition for anticancer comprising the quarternized amino glucosamine of claim 1 as effective component.

【Claim 3】

<59> The composition for anticancer according to claim 2 which is prepared in the form of dispersant, tablet, capsule, powders, ointment composition, solution, gel, paste, patch and granule.

【DRAWINGS】

【Figure 1】



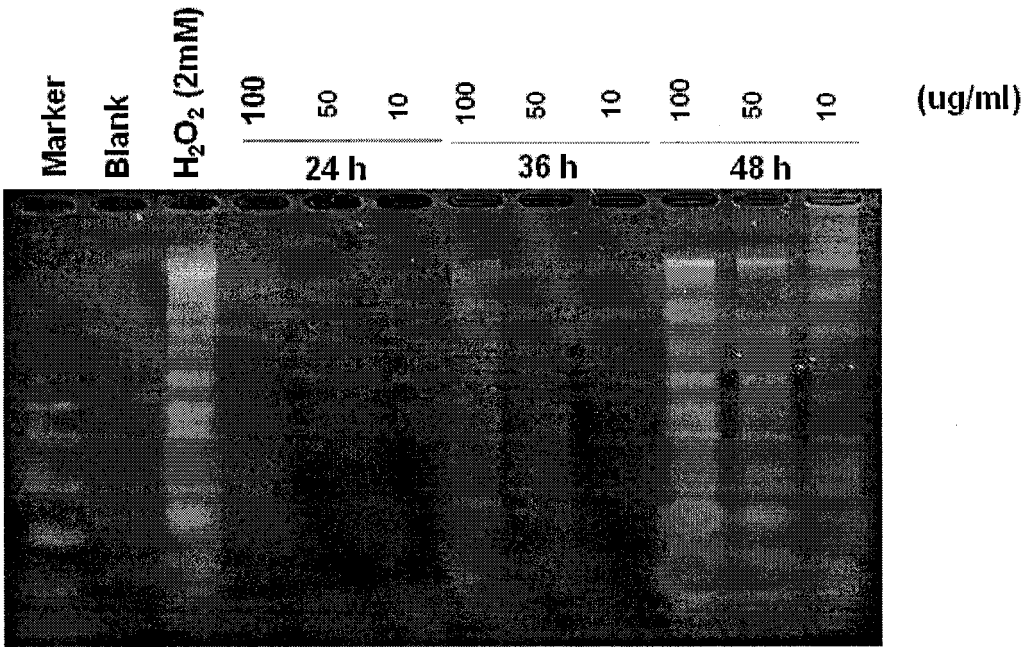
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【Figure 2】

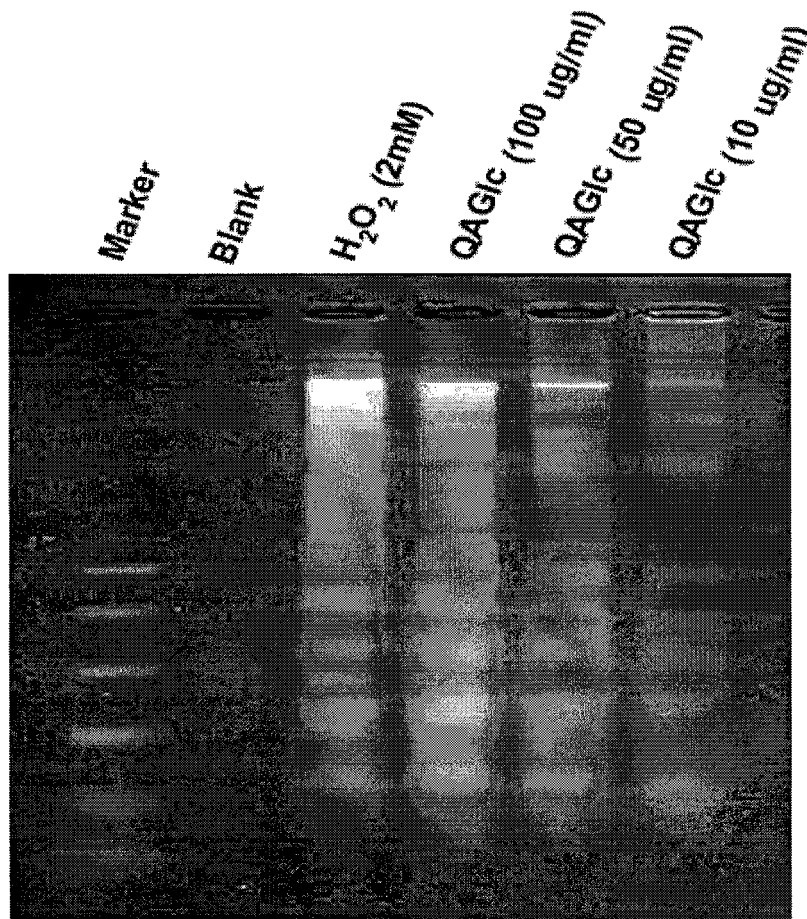
Concentration (%)	Anticancer Effect (%)				
	SW480	A549	B15F1	HL60	Hep3B
0.01	92.69±2.56	48.38±0.72	58.84±1.36	98.69±2.34	88.28±1.06
0.005	89.87±2.03	25.80±1.40	42.88±1.73	93.77±2.16	68.12±1.36
0.001	42.14±0.96	23.47±0.54	35.79±1.46	62.36±1.24	23.00±0.86
0.0005	29.81±1.06	2.24±0.23	13.74±0.76	46.07±1.51	21.17±0.58

<64>

【Figure 3】

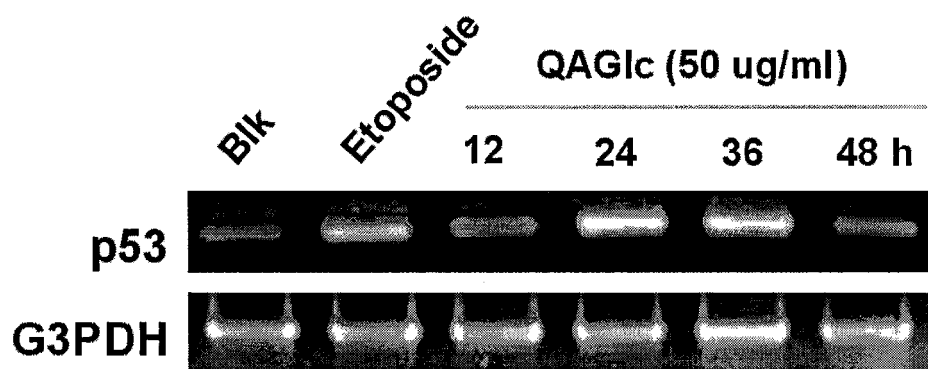


【Figure 4】



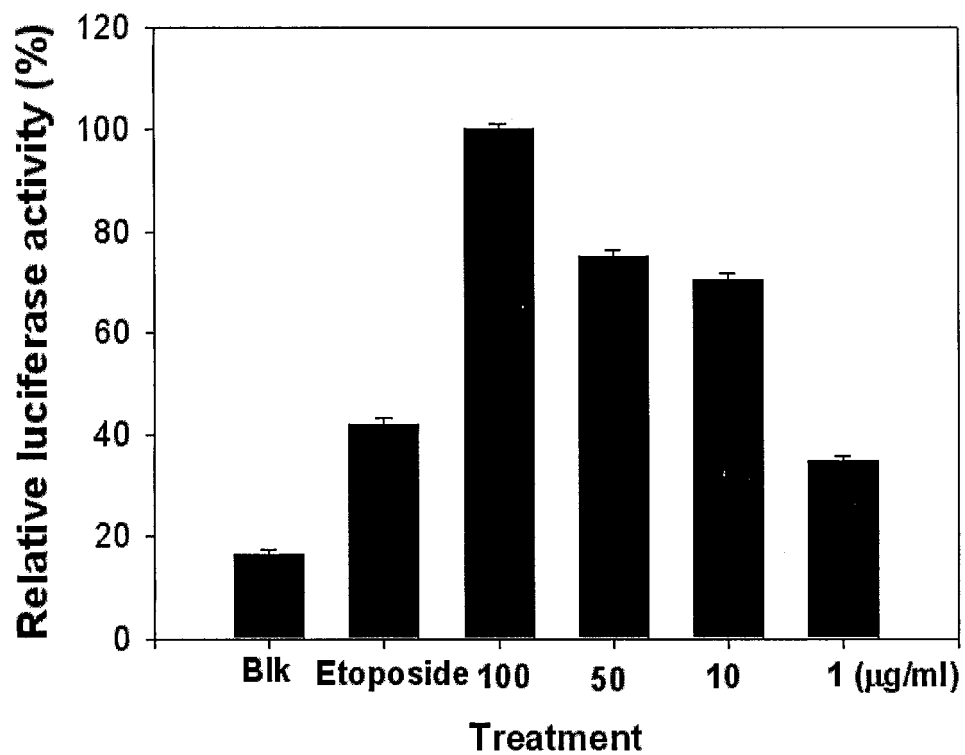
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【Figure 5】



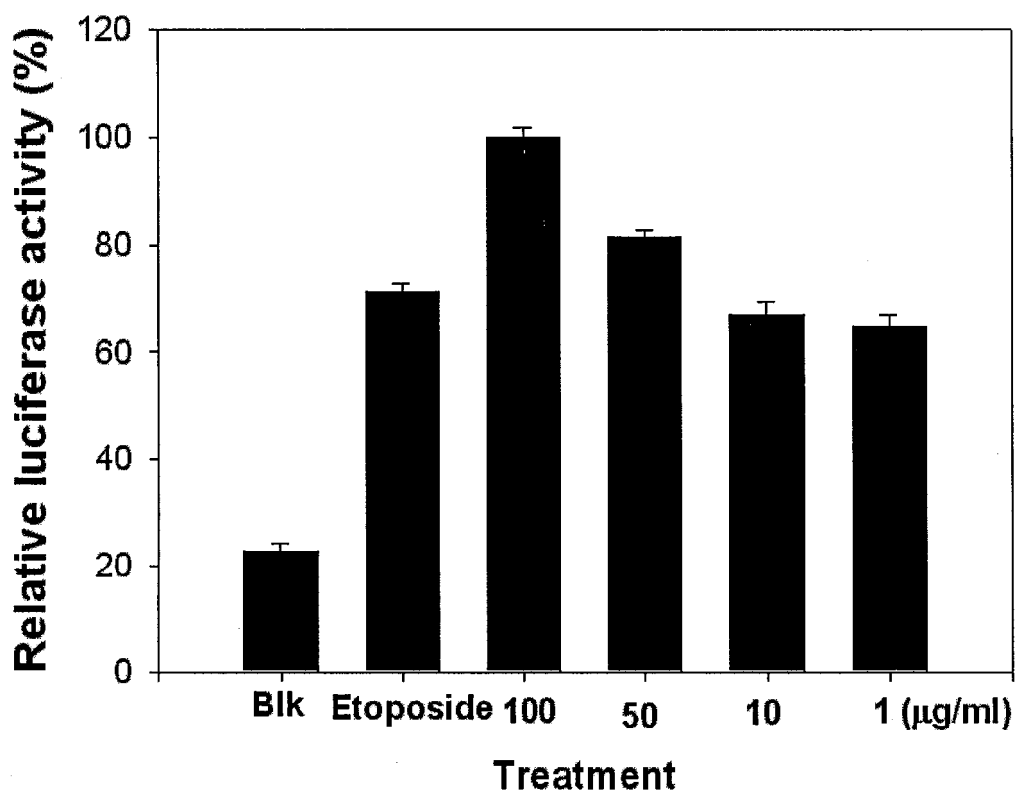
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【Figure 6】



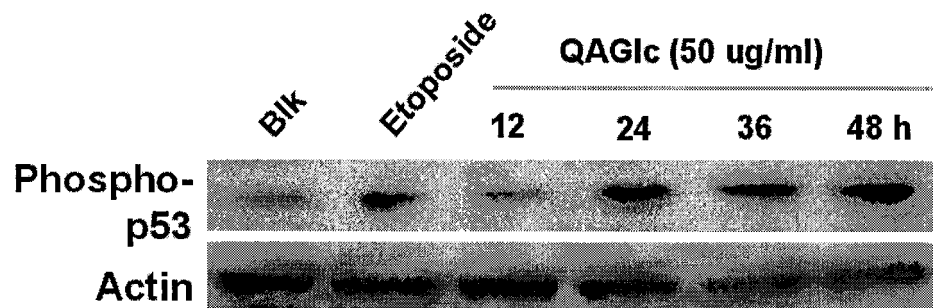
<68>

【Figure 7】



<69>

【Figure 8】



VIII-5-1	Declaration: Non-prejudicial disclosure or exceptions to lack of novelty Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name (LAST, First)	in relation to this international application KIM, Moon Moo declares that the subject matter claimed in this international application was disclosed as follows:
VIII-5-1(i)	Kind of disclosure:	other <OTHER: Symposium>
VIII-5-1(i)	Date of disclosure:	23 April 2006 (23.04.2006)
VIII-5-1(i)	Title of disclosure:	Quarternized amino glucosamine exerts anticancer effect on murine melanoma cells
VIII-5-1(i)	Place of disclosure:	Proceedings of the 7th Asia-Pacific Chitin and Chitosan Symposium

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/003671**A. CLASSIFICATION OF SUBJECT MATTER***C07H 5/06(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07H 5/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN(CASLINK)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 1548445 A (CHINA OCEAN UNIV.) 24 NOV. 2004, see entire document.	1 - 3
A	JP 08301768 A (TANABE SEIYAKU CO.) 19 NOV. 1996, see entire document.	1 - 3
A	WO 9514026 A1 (LABORATORIES OM S.A.) 26 MAY 1995, see entire document.	1 - 3
A	WO 9513061 A1 (IMMUNAL KFT.) 18 MAY 1995, see entire document.	1 - 3
A	SOSNOVSKY et. al. 'In the search for new anticancer drugs. 27. Synthesis and comparison of anticancer activity in vivo of amino acids, carbohydrates, and carbohydrate-amino acid conjugates containing the [N'-(2-chloroethyl)-N'-nitrosoamino]carbonyl Group' In: Journal of Pharmaceutical Sciences, 1994, 83(7), p.989-998	1 - 3

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search

28 MARCH 2007 (28.03.2007)

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2006/003671

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 1548445 A	24-11-2004	None	
JP 08301768 A	19-11-1996	None	
WO 9514026 A1	26-05-1995	US 6005099 JP 09505071 T2 EP 0729473 B1	21-12-1999 20-05-1997 23-08-2000
WO 9513061 A1	18-05-1995	JP 08508045 T2 EP 0679081 B1	27-08-1996 06-02-2002