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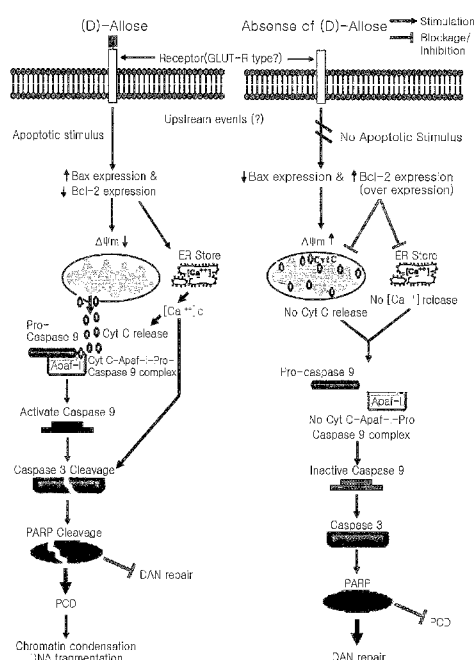
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(54) Title: (D)-ALLOSE INDUCING PROGRAMMED CELL DEATH IN HORMONE REFRACTORY PROSTATE CANCER LINES

[FIG. 5]



(57) Abstract: The present invention relates allose by inducing programmed cell death in hormone refractory prostate cancer cell lines. More particularly, the present invention relates to a D-allose inducing programmed cell death in hormone refractory prostate cancer cell line of DU 145 in dose-dependent manner by repressing cell growth and inhibiting cell cycle as well as by reducing expression of caspase-3 and poly (ADP-ribose) polymerase (PARP).

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【DESCRIPTION】**【Invention Title】**

(D)-ALLOSE INDUCING PROGRAMMED CELL DEATH IN
5 **HORMONE REFRACTORY PROSTATE CANCER LINES**

【Technical Field】

The present invention relates to D-allose inducing programmed cell death in hormone refractory prostate cancer cell lines. More particularly, the present invention relates to a D-allose inducing programmed cell death in hormone refractory prostate cancer cell line of DU145 in dose-dependent manner by repressing cell growth and inhibiting cell cycle as well as by reducing expression of caspase-3 and poly (ADP-ribose) polymerase (PARP).

15 **【Background Art】**

Because of high prevalence, mortality, and unsatisfactory treatment options available at this time, hormone-refractory prostate cancer (HRPC) becomes the second leading cause of cancer related death in men in the United States. In man, prostate homeostasis is dependent on the equilibrium between cell proliferation through cell division, and cell loss through apoptosis (programmed cell death, PCD). Defects in apoptosis signaling may hinder this balance, thereby enhancing tumor development and metastasis to distant sites like seminal vesicle, urinary bladder, rectum, bone, lymph node via blood and brain in a more aggressive form resulting in lethality. The metastatic prostate cancer (PC) is

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initially responsive to standard treatment regimes such as hormone therapy, radiotherapy, chemotherapy, and prostatectomy. However, in late stage, this metastatic disease is shifted from androgen dependence to androgen independence, which is often provoked by androgen-ablation therapy.

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The bcl-2 family of proteins (both anti- and pro-apoptotic members) is of special importance in this regards, as they can heterodimerize and seemingly titrate one another's function, thus controlling programmed cell death signaling pathway via their relative concentrations. These proteins function at a common
10 part of the apoptosis signaling pathway by acting as a checkpoint upstream of caspases, executors of apoptosis, and mitochondrial dysfunction. The apoptosis signaling pathway also considers mitochondrial permeability transition due to loss of mitochondrial transmembrane potential ($\Delta\psi_m$), and the release of cytochrome C (cyt C) as the central events initiating the execution of
15 programmed cell death.

Mitochondrial depolarization in apoptosis is thought to be associated with the permeability transition (PT); an event involving the formation of a voltage-dependent anion channel (VDAC) in the mitochondrial membranes,
20 which is also blocked by Bcl-2. The calcium concentration in cytosol ($[Ca^{2+}]_c$) is the prerequisite for post mitochondrial events involved in downstream caspase activation and subsequent induction of apoptosis in human prostate cancer cell line. The expression of anti-apoptotic bcl-2 is often enhanced in late stage HRPC that promotes cell survival by preventing interaction of Apaf-1-cyt C complexes,

result in caspases (caspase 9 to caspase 3) inactivation. Bcl-2 over expression due to recurrence of androgen independence further produces chemoresistant phenotype by genetic alterations. Therefore, new approaches for the management of HRPC are urgently needed.

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Rare sugars are defined by the International Society of Rare Sugars (ISRS) as monosaccharides that are rarely distributed in nature (The 1st International Symposium of ISRS, Takamatsu, Japan, 2002). Because of scarcity and cost, the biological function of rare sugars has not been investigated in detail, except their
10 usages as low-calorie carbohydrate sweeteners and bulking agents. However, after “Izumoring”, enormous research with rare sugars especially D-allose (C-3 epimer of glucose) has become possible. D-Allose inhibited segmented neutrophil production and lowered platelet count without any side effects, and may play a pivotal role in organ or tissue transplantation as an immunosuppressant. The novel
15 inhibitory effects of D-allose on the production of reactive oxygen species (ROS) from neutrophils and on the proliferation of various cancer cell lines were also reported. But, till today there is no report on the effect of D-allose on prostate cancer treatment, especially late stage HRPC.

20 **【Disclosure】****【Technical Problem】**

Therefore, the present inventors have made an effort on investigating the dose and time-dependent effects of D-allose on the proliferation of late stage human HRPC using DU145 cell line in vitro, which are highly tumorigenic and

chemotherapy-resistant. The present inventors confirmed the mechanism of D-allose induced apoptosis in DU145 cell line by modulation of pro- and anti-apoptotic Bcl-2 family members, Bax and Bcl-2, in favor of execution of programmed cell death, which is accompanied by mitochondrial cyt C release
5 along with concomitant alteration of mitochondrial $\Delta\psi_m$, elevation of $[Ca^{2+}]_c$, and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP), thereby completing the present invention.

【Technical Solution】

10 The present invention relates to estimate the dose and time-dependent effects of D-allose on the proliferation of late stage human HRPC using DU145 cell line in vitro, which are highly tumorigenic and chemotherapy-resistant. Also, the present invention relates to estimate the mechanism of D-allose induced apoptosis in DU145 cell line by modulation of pro- and anti-apoptotic Bcl-2 family
15 members, Bax and Bcl-2, in favor of execution of programmed cell death, which is accompanied by mitochondrial cyt C release along with concomitant alteration of mitochondrial $\Delta\psi_m$, elevation of $[Ca^{2+}]_c$, and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP).

20 【Advantage Effects】

It is an effect of the present invention to provide the dose and time-dependent effects of D-allose on the proliferation of late stage human HRPC using DU145 cell line in vitro, which are highly tumorigenic and chemotherapy-resistant. Also, it is an effect of the present invention to provide the

mechanism of D-allose induced apoptosis in DU145 cell line by modulation of pro- and anti-apoptotic Bcl-2 family members, Bax and Bcl-2, in favor of execution of programmed cell death, which is accompanied by mitochondrial cyt C release along with concomitant alteration of mitochondrial $\Delta\psi_m$, elevation of $[Ca^{2+}]_c$, and
5 cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP).

【Description of Drawings】

FIG. 1 shows effects of D-allose on cell growth, apoptosis and cell cycle distribution of human HRPC cell line, DU145. Cells are exposed to 0, 1, 5, 10, 20
10 and 40 mM of D-allose for up to 72 h and then assessed for cell viability (by MTT assay), programmed cell death (by DAPI/Annexin-V-FLUOS staining) and cell cycle distribution (by PI staining). Detail procedures are mentioned in Examples. (A) Growth inhibitory effects are expressed as percentage of cell growth inhibition induced by D-allose for 24, 48 and 72 h relative to the untreated
15 controls. Results represent the mean $\pm$ SEM of the three independent experiments. * P <0.05, ** P <0.01, † P <0.001. (B) Cells are in the culture media with and without D-allose treatments for 48 h $\times$ 400. (C) Net apoptosis induction by D-allose for 24, 48 and 72 h relative to the untreated controls. Results represent the mean $\pm$ SEM of the three independent experiments. * P <0.05,
20 ** P <0.01, † P <0.001. (D) Apoptotic cells by DAPI staining with and without D-allose treatments for 48 h $\times$ 400. The confocal images are the basis of calculation of apoptotic indices as mentioned in (C). (E) Flow cytometric analysis of apoptotic cells by annexin-V/PI staining upon treatments with D-allose for 48 and 72 h. Results are the true representative of the triplicate experiments. (F)

Distribution of D-allose treated cells for 48 h in the different phases of cell cycle. Percentage of cells in the G0–G1, G2–M and S phases are calculated with ModFit LT software, version 2.0 (Beckon Dickinson), and are presented in the right side of the histograms. Results are the true representative of the triplicate experiments.

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FIG. 2 shows effects of D-allose on Bcl-2, Bax, caspase 3 and PARP expression levels in human HRPC cell lines, DU145. Cells are exposed to 0, 20 and 40 mM of Dallose for 72 h. Detail procedures are mentioned in Examples. (A) After exposure cells are lysed and assayed for Bcl-2, Bax, full length caspase 3 and cleaved caspase 3, full length PARP and cleaved PARP proteins expression by Western blot analysis. β -actin expression indicates uniform loading in each lane. Results are the true representative of the triplicate experiments. (B) Densitometric evaluations of Bcl-2, Bax, full length caspase 3 and cleaved caspase 3, full length PARP and cleaved PARP protein bands of (A), respectively by Sigma Gel, version 1.0. Results represent the mean $\pm$ SEM of the three independent experiments. $*P<0.05$, $**P<0.01$, $^{\dagger}P<0.001$. (C) Cells with and without D-allose treatments are subjected to analysis of Bcl-2, Bax and caspase 3 mRNAs by RT-PCR, where β -actin is taken as loading control. Results are the true representative of the triplicate experiments. (D) Densitometric analysis of Bcl-2, Bax and caspase 3 mRNA bands of (C), respectively by Molecular AnalystTM, version 1.4.1 (Bio-Rad). Results represent the mean $\pm$ SEM of the three independent experiments. $*P<0.05$, $**P<0.01$, $^{\dagger}P<0.001$.

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FIG. 3 shows effects of D-allose on mitochondrial $\Delta\psi_m$, cyt C expression

and nuclear morphology in human HRPC cell lines, DU145. Cells are exposed to 0, 20 and 40 mM of D-allose for up to 72 h. Detail procedures are mentioned in Examples. (A) The immunofluorescence of DU145 cell line doublestained for cyt C (FITC-labeled cyt C antibody, green) and chromatin (PI, red) upon treatments with D-allose for 48 h. Yellow color (green + red; merge image) indicates release of cyt C from mitochondria into cytosol and chromatin condensation in the same cells, as detected by confocal microscopy $\times 600$ (0 mM), $\times 400$ (20 mM), $\times 3500$ (40 mM). DU145 cell line treated with FITC-labeled secondary antibody alone is considered as negative control $\times 600$. (B) The translocation of cyt C is observed in the confocal image of double-stained DU145 cell line after treatments with 40 mM of D-allose for 72 h. Mitochondria are probed with FITC-labeled cyt C antibody (green) and nucleus is counterstained with PI (red) $\times 3000$. The fluorescence micrographs of mitochondria (JC-1, green) in DU145 cell line with and without D-allose treatments for 72 h $\times 600$ (0 mM), $\times 450$ (20 and 40 mM). (C) Flow cytometric analysis of mitochondrial $\Delta\psi_m$ by JC-1 staining upon treatments with D-allose for 48 and 72 h. Loss of mitochondrial $\Delta\psi_m$ is associated with increase in FL1 fluorescence. Results are the true representative of the triplicate experiments. (D) Quantification of cells with low mitochondrial $\Delta\psi_m$ (as the percentage of total cell population) induced by D-allose for 24, 48 and 72 h, as detected by flow cytometry. Results represent the mean $\pm$ SEM of the three independent experiments. * $P < 0.05$, ** $P < 0.01$, $^{\dagger}P < 0.001$.

FIG. 4 shows effects of D-allose on $[Ca^{2+}]_c$ in human HRPC cell lines, DU145. Cells are exposed to 0, 20 and 40 mM of D-allose for 48 h followed by fura-2 AM labeling and fluorescence spectra for $[Ca^{2+}]_c$ are measured by

luminescence spectrophotometer. Detail procedures are mentioned in Examples.
Inset: The fluorescence micrographs of fura-2 AM labeled single DU145 cell with
(A, 20 mM) and without (B, 0 mM) D-allose treatments for 48 h $\times 2500$.

5 FIG. 5 shows proposed mechanism of D-allose as an antiproliferative agent
mediates through induction of programmed cell death in late stage human HRPC
cell line, DU145. D-allose in vitro exposure to human HRPC cell lines could alter
Bcl-2/Bax ratio that decreases mitochondrial $\Delta\psi_m$ and promotes cyt C release from
mitochondria leading to activation of downstream caspase 3 and finally cleavage of
10 the caspase 3-target protein PARP, result in chromatin condensation, a hallmark of
programmed cell death. High $[Ca^{2+}]_c$ is the earliest biochemical event of this
mitochondria mediated intrinsic pathway of apoptosis. On the other hand, Bcl-2
over expression prevents leakage of Ca^{2+} from its cellular ER store, lowers $[Ca^{2+}]_c$,
subsequently stops cyt C release from mitochondria, and ultimately blocks
15 apoptosis (programmed cell death). However, it would be interesting to investigate
in future whether D-allose has any role in the very upstream events of intrinsic
pathway of apoptosis in HRPC cell lines involving DU145.

【Best Mode】

20 The object of the present invention is to provide D-allose for the
induction of apoptosis and inhibition of proliferation in hormone refractory
prostate cancer cell lines of DU145 in vitro.

Further, said D-allose induces apoptosis in hormone refractory prostate

cancer cell lines by i) lowering the mitochondrial transmembrane potential ($\Delta\psi_m$);
ii) releasing the cytochrome C (cyt C); iii) cleaving the caspase 3 and poly
(ADP-ribose) polymerase (PARP); and iv) elevating the calcium concentration in
cytosol ($[Ca^{2+}]_c$).

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Further, said D-allose induces apoptosis in hormone refractory prostate
cancer cell lines by modulating expression at the transcription level of
apoptosis-related genes through increasing expression of pro-apoptotic family,
Bax and decreasing expression of anti-apoptotic family, Bcl-2.

10

Further, the other object of the present invention is to provide an effective
dose of D-allose is 20~40 mM per 1×10^5 cells of DU145 cell line.

Development of effective agents for treatment of hormone-refractory
15 prostate cancer (HRPC) has become a national medical priority. D-Allose is a
monosaccharide (C-3 epimer of glucose) distributed rarely in nature; because of
its scarcity and cost, the biological effect has hardly been studied. In the present
invention, we demonstrated the inhibitory action of D-allose on proliferation of
human HRPC cell lines, DU145 in a dose- and time-dependent manner.

20

In vitro treatment of D-allose resulted in the alteration of Bcl-2/Bax ratio
in favor of apoptosis (programmed cell death, PCD) in the DU145 cell line,
which was associated with the lowering of mitochondrial transmembrane
potential ($\Delta\psi_m$) and the release of cytochrome C (cyt C), the cleavage of caspase

3 and poly (ADP-ribose) polymerase (PARP), and the elevation of calcium concentration in cytosol ($[Ca^{2+}]_c$).

D-Allose also induced G1 phase arrest of the cell cycle in DU145 cell line. This invention for the first time suggested the antiproliferative effect of
5 D-allose through induction of programmed cell death in HRPC cell line, DU145 which could be due to the modulation of mitochondria mediated intrinsic apoptotic pathway.

Hereinafter, the present invention explains in detail.

10

D-Allose inhibited cell growth in DU145 cell line

The aim of the present invention was to investigate whether rare sugar D-allose imparts anti-proliferative effects against human HRPC cell line. Therefore, DU145 cell line was treated with different concentrations of D-allose
15 and cell growth was assayed by MTT assay. D-Allose treatment (0–40 mM) resulted in a significant dose-dependent inhibition of cell growth in DU145 (FIG. 1A, B) cell line as compared to the control. D-Allose treatment also resulted in time-dependent inhibition of DU145 cell growth, which was more pronounced at 72 h post-treatment in contrast with the control (FIGs. 1A). We also examined the
20 effect of D-allose on the growth of human NPE cell line, PrEC.

Our results revealed PrEC cell line showed no remarkable effect. We further studied the effect of D-glucose on proliferation of the DU145 cell line, which revealed significant increase of cell growth in a dose-dependent manner.

D-Allose induced programmed cell death in DU145 cell line

In order to determine whether D-allose-induced growth inhibition is due
5 to programmed cell death, we evaluated the apoptotic indices at the indicated
times of treatment (i.e. 24, 48 and 72 h) by microscopical analysis. For this
purpose DNA intercalating dye DAPI was used on human HRPC cell line,
DU145 and NPE cell line, PrEC. As expected, very negligible effect was
observed in PrEC cell line in contrast with the other cancer cell line studied (FIG
10 1C, D). D-Allose induced significant dose- and time-dependent programmed cell
death in DU145 (FIG. 1C) cell line, as revealed by DAPI staining.

The extent of apoptosis was quantified by flow cytometric analysis where
DU145 cell line was labeled with annexin-V and PI. In DU145 cell line, D-allose
15 of 20 and 40 mM caused 5.1% and 8.7% of apoptotic cells in 48 h treated groups,
and 11.9% and 23.5% of apoptotic cells in 72 h treated groups, as compared to
the respective untreated controls i.e. 1.2% and 2.3% of apoptotic cells in 48 and
72 h control groups, respectively (FIG. 1E). Therefore, induction of apoptosis
(programmed cell death) was significantly higher when high dose of D-allose (40
20 mM) was applied for 72 h. Our invention also revealed no remarkable effect of
D-glucose on the programmed cell death in DU145 cell line.

D-Allose induced G1 phase arrest of cell cycle in DU145 cell line

To assess whether D-allose induced growth inhibition and apoptosis is mediated through the alterations in cell cycle, the cell cycle analysis of DU145 cell line was performed after treatments with various concentrations of D-allose for 48 and 72 h. Compared with the untreated control (31.9% cells in G0–G1 phase), D-allose treatment for 48 h showed significant arrest of cells in G0–G1 phase of the cell cycle (48.7% cells at 20 mM concentration that further increased to 57.5% at 40 mM concentration) (FIG. 1F). Further, the remarkable increase in G0–G1 cell population was accompanied with a concomitant decrease of cell number in G2–M phase and S phase of the cell cycle (FIG. 1F). D-Allose treatment for 72 h also maintained the similar trend (data not shown).

D-Allose altered Bax and Bcl-2 protein expression in DU145 cell line

D-Allose altered Bax and Bcl-2 proteins expression in DU145 cell line

Pro-apoptotic Bax and anti-apoptotic Bcl-2 are known to play a crucial role in apoptosis; therefore, the dose-dependent effect of D-allose on protein levels of Bax and Bcl-2 in DU145 cell line was studied. The Bcl-2 protein expression was significantly decreased by D-allose treatment at 20 and 40 mM concentrations for 72 h, while Bax protein expression was remarkably increased at 40 mM concentration of D-allose under the same condition in DU145 (FIG. 2A, B) cell line. Overall, the treatment of cells with D-allose resulted in a remarkable dose-dependent shift (i.e. 0.896 at 0Mm, 0.335 at 20Mm, 0.126 at 40Mm) in Bcl-2/Bax ratio, suggesting induction of programmed cell death in human HRPC cell line, DU145.

D-Allose altered Bax and Bcl-2 mRNAs expression in DU145 cell line

To demonstrate that up-regulation of Bax and Bcl-2 proteins in DU145
5 cell line by D-allose correlated with an alteration in the amount of Bax and Bcl-2
mRNAs, RT-PCR analysis was performed. As shown in FIG. 2C and D, the
treatment D-allose in DU145 cell line for 72 h significantly reduced Bcl-2 mRNA
expression in a dose-dependent manner, whereas Bax mRNA expression showed
the opposite effect. Also, the expression pattern of both the pro- and
10 anti-apoptotic mRNAs in DU145 cell line exhibited the similar trend with that of
the corresponding protein levels, confirming the role of D-allose during induction
of programmed cell death in the human HRPC cell line at the transcript level of
programmed cell death-related genes, Bax and Bcl-2.

D-Allose induced cyt C release and alteration of mitochondrial $\Delta\psi_m$ with subsequent cleavage of caspase 3 and PARP

The process of programmed cell death may involve disruption of
mitochondrial function through the release of cyt C from mitochondria, which
20 interacts with Apaf-1 leading to activation of caspases and subsequent cleavage
of PARP; therefore, the dose-dependent effect of D-allose on caspase 3 and
PARP protein levels in DU145 cell line were studied. As shown in FIG. 2A and
B, the different concentrations of D-allose (0, 20 and 40 mM) for 72 h resulted in
a remarkable dose-dependent decrease in the full length caspase 3 and PARP

proteins expression with concomitant increase of their cleaved products in DU145 cell line. Also, D-Allose treatment for 72 h further revealed the similar type of dose-dependent effect on caspase 3 mRNA expression in DU145 cell line (FIG. 2C, D), as compared to the corresponding protein level (FIG. 2A, B).

5

The dose- and time-dependent effects of D-allose on the release of cyt C from mitochondria and condensed chromatin in DU145 cell line were well established in the present invention by immunofluorescence technique (FIG. 3A, B). The confocal laser microscopy revealed a diffuse staining pattern of cyt C, suggesting its release from mitochondria (cyt C, green) along with chromatin condensation (PI, red) upon treatments with different concentrations of D-allose for 48 h (FIG. 3A). The merge image (red + green = yellow) indicated the release of cyt C from mitochondria into cytosol and chromatin condensation as a result of D-allose treatment in the same DU145 cell line (FIG. 3A). Further, the translocation of cyt C was observed in the confocal image of double-stained DU145 cell line after treatments with 40 mM of D-allose for 72 h, where mitochondria were probed with an FITC-labeled cyt C antibody (green) and nucleus was counterstained with PI (red) (FIG. 3B).

20

Since Bcl-2 is located on the mitochondrial membrane and involved in the regulation of PT and the cyt C release, the present inventors also examined the mitochondrial $\Delta\psi_m$ in D-allose treated DU145 cell line for 24, 48 and 72 h by use of fluorescent dye JC-1. Uptake of JC-1 into mitochondria was monitored by flow cytometry, where loss of mitochondrial $\Delta\psi_m$ was associated with increase in

FL1 fluorescence (FIG. 3C). As shown in FIG. 3C and D, treatment of DU145 cell line with D-allose resulted in the appearance of a remarkable subpopulation of cells with low mitochondrial $\Delta\psi_m$ in a dose- and time-dependent manner. Further, the fluorescence micrographs of JC-1 stained mitochondria (green) in DU145 cell line with and without D-allose treatments for 72 h were supported by the flow cytometrical data shown in FIG. 3C and D.

D-Allose increased $[Ca^{2+}]_c$ in DU145 cell line

$[Ca^{2+}]_c$ is the prerequisite for post-mitochondrial events including caspase activation and subsequent programmed cell death in human prostate cancer cell line. The dose-dependent effect of D-allose on $[Ca^{2+}]_c$ for 48 and 72 h was considered in the present invention. As shown in FIG. 4A, $[Ca^{2+}]_c$ was remarkably elevated in DU145 cell line at 20 and 40 mM concentrations of D-allose for 48 h. These results were further confirmed by the fluorescence micrographs of fura-2 labeled single DU145 cell. D-Allose treatment for 72 h also maintained the similar trend (data not shown). Overall, the dose- and time-dependent effect of D-allose suggesting the induction of programmed cell death in human HRPC cell line, DU145 that was triggered by the increase of $[Ca^{2+}]_c$.

The search for promising agents that could reduce the incidence and burden of cancer has become increasingly important in recent years. Immense interest has been generated for rare sugars especially D-allose in view of the role

in attenuating the risk of developing cancer. The role of D-allose on HRPC needs to be addressed as PC responds initially to androgen ablation and later converts to hormone-resistant state. The recurrence of androgen independence often leads to alterations of several programmed cell death -related genes; results in anti
5 apoptosis and chemoresistance. In this regard, DU145 cells are unique in vitro models of late stage HRPC that are highly resistant to chemotherapeutic agents. By using DU145 cell line, the present inventors showed the potentiality of D-allose to inhibit cellular growth in dose- and time-dependent manner. In contrast, D-glucose remarkably increases cell growth without any prominent
10 effect on the programmed cell death in the human HRPC cell line, DU145. It is already known that some monosaccharides govern many cellular functions including strong inhibition on cell proliferation either in vivo or in vitro conditions because of their sugar structure. Since D-allose is the C-3 epimer of D-glucose, therefore, in the light of above observations it can be said that the
15 novel anti-proliferative effect of D-allose on human HRPC cell lines might be associated with its sugar structure.

Whether D-allose-induced growth inhibition is due to programmed cell death, the present inventors next evaluated apoptosis in DU145 cell line.
20 Apoptosis is a physiological process by which cells are removed when an agent damages their DNA. Apoptosis represents a discrete manner of programmed cell death that differs from necrosis and is regarded as an efficient way to eliminate damaged cells. Agents that can modulate apoptosis may be able to affect steady-state cell population, which may be useful in cancer therapy. The data of

the present invention demonstrated that D-allose induces significant dose- and time-dependent programmed cell death in DU145 cell line, which was remarkably higher when high dose of D-allose was applied for 72 h. This observation verified by chromatin condensation, Bcl-2/Bax ratio, caspase 3 and
5 PARP cleavage, fluorescence microscopy, and flow cytometry. This is the most important finding of the present invention since modulation of apoptotic response finally suggests a novel mechanism based therapeutic approach towards late stage HRPC.

10 As cell cycle regulation and apoptosis are closely associated phenomena, in next series of experiment the present inventors analyzed the cell cycle phase distribution of DU145 cell line by flow cytometry. Compared with the untreated controls, D-allose treatment resulted in remarkable dose- and time-dependent increase of cell numbers in G0–G1 phase with a concomitant decrease of cell
15 population in G2–M and S phase of the cell cycle, suggesting D-allose induce antiproliferative effect and programmed cell death in DU145 cell line involves cell cycle arrest at G1 phase.

Since sequential expression and activation of cyclins, cdks and cdk
20 inhibitors play a crucial role in the regulation of eukaryotic cell cycle, therefore involvement of cyclin-cdk machinery during induction of cell cycle arrest and programmed cell death by D-allose needs to be studied further.

The Bcl-2 family members, pro-apoptotic Bax and anti-apoptotic Bcl-2

are the critical regulators of apoptotic pathway. Bcl-2 has been found in very high levels in many human tumors and carcinoma cells, over expression of which results in resistance to apoptosis that may lead to chemoresistance. Bcl-2 forms a heterodimer with Bax and neutralizing its effects. Also, the PC marker, Bcl-2/Bax ratio dictates a cell's susceptibility to undergo apoptosis under various experimental conditions. In the present invention, a decrease in Bcl-2 protein with consequent increase in Bax protein expression in DU145 cell line after D-allose treatment caused a significant dose-dependent shift in Bcl-2/Bax ratio towards induction of programmed cell death. Further, in the present invention the expression of Bax and Bcl-2 mRNAs maintain the similar trend with that of their corresponding proteins, suggesting up regulation of Bax and Bcl-2 proteins occur at the transcript level during induction of programmed cell death in DU145 cell line by D-allose. However, the result obtained in this invention that D-allose induce apoptosis is mediated through Bcl-2 family of proteins, Bax and Bcl-2, is in contrast with the previous studies, which may be due to the differences in experimental design and cancer cell line used.

Previous studies showed that alteration in the Bcl-2/Bax ratio promotes cyt C release from mitochondria, which activates Apaf-1, allowing it to bind to and activate caspase 9. Caspase 9 then cleaves and activates downstream caspase 3, resulting in apoptosis. Alternatively, it is possible that the lack of caspase 9 activation could result from the failure to interact with Apaf-1-cyt C complexes, as influenced by Bcl-2 over expression. On the other hand, due to its unique location on mitochondrial membrane, Bcl-2 stabilizes the mitochondrial $\Delta\psi_m$ and

inhibits cyt C translocation from the intermembrane space to cytosol, result in blockage of downstream caspase 3 activation and subsequent programmed cell death process.

5 In the present invention, the dose-dependent shift in Bcl-2/Bax ratio towards induction of programmed cell death in DU145 cell line after D-allose treatments is associated with mitochondrial cyt C release and a decrease in $\Delta\psi_m$, suggesting opening of the mitochondrial PT pore. These events are linked to the VDAC, a component of the PT pore, and its interaction with Bax. Since Bcl-2
10 functionally inactivate Bax by heterodimerization, decrease expression of Bcl-2 in DU145 cell line by D-allose treatment lowers the threshold at which the execution of apoptosis occurs.

 This whole event is further associated with the appearance of condensed
15 chromatin in D-allose treated DU145 and cell line, a hallmark of apoptosis usually succeeding mitochondrial events. Activation of caspase 3-like protease, which is generally involved in the degradation of death substrates like PARP and constitutes the execution phase of programmed cell death controlled by Bcl-2, is also well established in the present invention.

20

 Further, in the present invention DU145 cell line revealed dose-dependent elevation of $[Ca^{2+}]_c$ upon D-allose treatments. Studies from other laboratories have shown that poor control of Ca^{2+} homeostasis leads to moderate rise in $[Ca^{2+}]_c$, result in apoptotic cell death. In human prostate cancer cell line,

the release of $[Ca^{2+}]_c$ regulates cyt C secretion, which further plays a role in post-mitochondrial events involved in downstream caspase 3 activation leading to induction of programmed cell death. On the other hand the anti-apoptotic protein Bcl-2 prevents the leakage of Ca^{2+} from its store in endoplasmic reticulum (ER),
5 thereby regulates $[Ca^{2+}]_c$. Therefore, experimental data as mentioned above, indicating the execution of apoptotic cell death in human HRPC cell line, DU145 through mitochondria mediated cyt C-caspase 3-PARP pathway is triggered by an increase in $[Ca^{2+}]_c$.

10 To understand the antiproliferative effect of D-allose through induction of programmed cell death in human HRPC cell line, DU145, the summary of the present invention is schematically represented in FIG. 5. Therefore, results of the present invention showed that D-allose inhibits DU145 cell growth by induction of programmed cell death through the Bcl-2 family proteins, Bax and Bcl-2, and
15 the mitochondria mediated intrinsic apoptotic pathway members like cyt C, caspase 3 and PARP.

The elevation of $[Ca^{2+}]_c$ plays a crucial role during early commitment of this intrinsic apoptotic pathway. Further, D-allose revealed no remarkable
20 cytotoxic effect on human NPE cell line, PrEC. Therefore, the present inventors finally suggest the role of D-allose as a promising antiproliferative agent in late stage PC, may be without any side effects on normal prostate cells. This is the first estimate that D-allose induces programmed cell death in human HRPC cell lines in vitro. Further mechanism-based in vivo studies on anticancer activities

with D-allose in animal models as well as in prostate biopsy samples are warranted.

Hereinafter, the preferred Examples are provided for better understanding.

5 However, these Examples are for illustrative purpose only, and the invention is not intended to be limited by these Examples.

【Mode for Invention】

Example 1: Cell culture and treatments

10

DU145 (human HRPC cell line, derived from a patient with brain metastasis) and PrEC (human normal prostate epithelial cell line) cell lines were obtained from Korea Cell Line Bank (KCLB, Seoul, South Korea). DU145 and PrEC cell lines were grown in triplicate culture plates containing RPMI-1640
15 (HyClone, Logan, UT, USA) and Dulbecco's Modified Eagle's Medium (HyClone, Logan, UT, USA) respectively, and kept in a humidified atmosphere containing 5% CO₂ at 37°C for 72 h. The media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 1% antibio-antimicotic solution containing 10,000 units/ml of penicillin and 10 mg/ml of streptomycin (HyClone,
20 Logan, UT, USA), HEPES, sodium bicarbonate, *p*-aminobenzoic acid and insulin (complete culture media). D-Allose was purchased from Sigma (St. Louis, MO, USA) and dissolved at a concentration of 1 M in sterile phosphate-buffered saline (PBS), and was further sterilized by filtration. To examine the dose- and time-dependent effects, the cell lines (70–80% confluent) were treated with 0–40

mM of D-allose for up to 72 h, whereas without D-allose treated cell lines (0 mM) were considered as control. DU145 cell line was also treated with 0–40 mM of D-glucose. All the other chemicals used in the work were procured from Sigma (St. Louis, MO, USA), unless otherwise specified.

5

Example 2: Cell viability

The logarithmic growth phase of DU145 and PrEC cell lines were taken for growth assay using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT). The cell lines were seeded onto 96-well plates with 1×10^5 cells/well in 200 μl of complete culture media containing 0, 1, 5, 10, 20 and 40 mM of D-allose and D-glucose for 24, 48 and 72 h. After incubation for the specified time at 37°C in a humidified 5% CO₂ incubator, cell viability was determined. MTT (5 mg/ml in PBS) was added to each well and incubated for additional 4 h at 37°C. To achieve solubilization of formazan crystal formed in viable cells, DMSO was added to each well, and the absorbance was recorded on a microplate reader at 595 nm wavelength. The effect of sugars on growth inhibition was assessed as percent cell viability where control cells were taken as 100% viable.

20

Example 3: Detection and quantification of apoptosis

The Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for the detection and quantification of the

apoptotic cells. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin-V (green fluorescence) and the necrotic cells are stained with propidium iodide (PI, red fluorescence). Briefly, DU145 cell line was grown at a density of 1×10^6 cells in triplicate culture plates and then treated with D-allose (0, 20 and 40 mM) for 48 and 72 h. The cells were treated with trypsin and harvested with PBS and processed for labeling with annexin-V and PI by the use of the kit according to the manufacturer's protocol. The fluorescence was measured by flow cytometric analysis, performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) using 488 nm excitation and a 515 nm band pass filter for fluorescein detection and a filter[600 nm for PI detection (FL1: Annexin-V-FLUOS; FL2: PI). Further, apoptotic indices were measured by analysis of fragmented or scattered nuclei and condensed chromosome as the stringent morphological criteria of apoptosis, detectable by confocal microscopy on cells stained with DNA-intercalating dye 4-6-diamino-2-phenylindole (DAPI). For this purpose, DU145 and PrEC cell lines were exposed to 0, 20 and 40 mM of D-allose for up to 72 h, stained with DAPI (1 μ g/ml in methanol), mounted with Prolong Antifade reagent (Molecular Probes, Eugene, OR, USA), and images were acquired by use of a confocal laser scanning microscope (Fluoview FV 1000, Olympus, Japan). The numbers of apoptotic nuclei per 100 cells were counted from the five high power fields/slide and apoptotic indices were expressed as a percentage of the untreated controls in each cell line, considering the value as 100%. DU145 cell line was also treated with 0–40 mM of D-glucose for determination of apoptotic indices as mentioned above.

Example 4: Cell cycle analysis

DU145 cell line (1×10^6 cells in triplicate culture plates) was treated with D-allose (0, 20 and 40 mM) for 48 and 72 h. The cells were treated with trypsin and harvested with PBS, centrifuged and resuspended the pellet in cold PBS-methanol mixture (1:9) for 1 h at 4°C. The cells were centrifuged, pellet washed and resuspended with PBS again, and incubated with RNase (20 mg/ml in PBS) for 30 min at 37°C. The cells were chilled over ice and stained with PI (1 mg/ml in PBS) for 1 h and analyzed by flow cytometry using CellQuest software, version 3.0, performed on a FACSCalibur, fluorescence (FL2-A) detector equipped with 488 nm argon laser light source and 623 nm band pass filter (Becton Dickinson, San Jose, CA, USA). Total 10,000 cells were acquired for analysis by CellQuest software and the quantification of FACS data was performed using ModFit LT software, version 2.0, (Becton Dickinson, San Jose, CA, USA). The cell population was gated according to the characteristics side scatter and forward scatter intensities of FACS. The number of all gated living cells was taken as 100%, from which the percentage of cells in G0-G1, S and G2-M phase was calculated and presented in the right side of the histogram.

Example 5: Protein isolation and Western blot analysis

After 0, 20 and 40 mM of D-allose treatments for 72 h, DU145 cell line (1×10^6 cells in triplicate culture plates) was harvested into cell lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1 mM PMSF. The cells were

then sonicated, centrifuged and protein concentration was measured by Bradford assay with the Bio-Rad protein assay solution using bovine serum albumin (BSA) as standard. Proteins (30 μ g) were separated on a 12.5% SDS-PAGE under reducing conditions and transferred onto a PVDF membrane (Santa Cruz
5 Biotechnology, Santa Cruz, CA, USA). Prestained protein marker, broad range: 6–175 kDa (New England Biolabs Inc., Ipswich, MA, USA) run in parallel for detection of the molecular weights of the proteins. Membrane was blocked with 5% (w/v) skimmed milk in order to reduce non-specific binding and immunoblotting was performed using rabbit derived anti-Bcl-2, anti-Bax, anti-Caspase 3 and
10 anti-PARP antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti- β -actin antibody (1:500; Sigma, St. Louis, MO, USA) was taken as control to confirm uniform loading. Membrane was probed with a goat derived horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and proteins were detected by the enhanced chemiluminescence
15 (ECL) detection system according to the vendor's protocol (Amersham Biosciences, Piscataway, NJ, USA) followed by X-ray exposure. The densitometry analysis of the protein bands were performed by Sigma Gel, version 1.0 (Jandel Scientific, San Rafeal, CA, USA).

20 Example 6: Calculation of Bcl-2/Bax ratio

Bcl-2/bax ratios of D-allose treated groups were calculated on the basis of the densitometric analysis of the corresponding protein bands and compared the values with that

of the untreated control.

Example 7: RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

5

To identify the relative levels of expression of Bcl-2, Bax and Caspase 3 mRNAs after 0, 20 and 40 mM of D-allose treatments for 72 h, RT-PCR was performed. DU145 cell line (1×10^6 cells in triplicate culture plates) was harvested as mentioned above and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following a standard laboratory protocol. The RNA purity and concentrations were determined. Then 2 μ g of total RNA was reversely transcribed to single-stranded cDNA using Oligo(dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA, USA) with M-MLV reverse transcriptase (Promega, Madison, WI, USA). For PCR reaction, 4 μ l of cDNA was reacted with 20 pmol each of the forward and reverse primers (Bioneer Corporation, Seoul, South Korea), and GoTaq[®] Green Master Mix 29 containing PCR buffer, 25 mM magnesium chloride, 10 mM dNTP mix and Taq enzyme (Promega, Madison, WI, USA), and amplified. The human primers of each transcript were as follows: Bcl-2 [5'-CGACGACTTC TCCCGCCGCTACCGC-3' (forward) (SEQ ID NO: 1) and 5'-CCGCATGC TGGGGCCGTACAGTTCC-3' (reverse) (SEQ ID NO: 2)], Bax [5'-GTGCACCAAGGTGCCGGAAC-3' (forward) (SEQ ID NO: 3) and 5'-TCAGCCCA TCTTCTTCCAGA-3' (reverse) (SEQ ID NO: 4)], Caspase 3 [5'-AGTGCTCGCAGCTCATACCT-3' (forward) (SEQ ID NO: 5) and 5'-GAGTCCATTGATTCGCTTCC-3' (reverse) (SEQ ID NO: 6)], and β -actin (as

loading control) [5'-GTGGGGCGCCCCAGGCACCA-3' (forward) (SEQ ID NO: 7) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse) (SEQ ID NO: 8)]. The conditions for PCR were as followings: initial denaturation at 94°C for 5 min; 25 cycles of denaturation at 94°C for 1 min, annealing for 1 min (Bcl-2: 68°C, Bax: 53°C, Caspase 3: 55°C, and β -actin: 63°C) and elongation at 72°C for 1 min; and another final extension step at 72°C for 10 min on a PC-812 Thermal Cycler (Astec, Fukuoka, Japan). The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide (1 μ l/ml in PBS) for 25 min and exposed to UV lamp for photography of the bands. The molecular sizes of the amplified products were determined by comparison with the molecular weight marker (100 bp DNAladder; Promega, Madison, WI, USA) run parallel with the PCR products. The densities of mRNA bands were analyzed by Molecular AnalystTM, version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Example 8: Visualization of mitochondrial cyt C release and nuclear morphology

The in situ analysis of cyt C was carried out by immunofluorescence technique. Briefly, DU145 cell line (1×10^6 cells in triplicate culture plates) was treated with D-allose (0, 20 and 40 mM for 48 and 72 h) and was fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton and washed with PBS in chilled condition. Cyt C was detected by using mouse anti-cyt C antibody and rabbit anti-mouse FITC-labeled antibody (1:250 and 1:200, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). DU145 cell line treated with FITC-labeled

secondary antibody alone was considered as negative control. Subsequently, chromatin was stained with PI (1 mg/ml in PBS) for 20 min in dark and slides were mounted with Prolong Antifade reagent (Molecular Probes, Eugene, OR, USA). Cyt C (green) and chromatin (red) staining patterns were acquired by use of a
5 confocal laser scanning microscope (Fluoview FV 1000, Olympus, Japan).

Example 9: Measurement of mitochondrial $\Delta\psi_m$

Mitochondrial $\Delta\psi_m$ was measured by JC-1 mitochondrial membrane
10 potential detection kit (Biotium Inc, Hayward, CA, USA) according to the manufacturer's protocol. Briefly, DU145 cell line (1×10^6 cells in triplicate culture plates) was harvested after treatments with 0, 20 and 40 mM of D-allose for up to 72 h as mentioned above, stained with 19 JC-1 reagent at 37°C for 15 min and resuspended twice in $1 \times$ assay buffer. Changes in mitochondrial $\Delta\psi_m$ were
15 measured at the single cell level by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) under the following conditions: FL1, 511 volts; FL2, 389 volts; FL1 – 10.5% FL2; FL2 – 25.9% FL1; 488 nm argon excitation laser and 585 nm band pass filter. In total 10,000 cells were acquired for analysis by CellQuest software, version 3.0 (Becton Dickinson, San Jose, CA, USA), and
20 the quantification of cells with low mitochondrial $\Delta\psi_m$ as the percentage of total cell population was performed. Further, the DU145 cell line was seeded onto chamber slides with 1×10^5 cells/chamber in 500 μl of complete culture medium containing 0, 20 and 40 mM of D-allose for 72 h, permeabilized with 0.1% Triton, stained with $1 \times$ JC-1 reagent in dark, mounted with Prolong Antifade reagent

(Molecular Probes, Eugene, OR, USA), and images (JC-1, green) were acquired by use of a fluorescence microscope (Axioplan 2, Carl Zeiss, Germany).

Example 10: Fura-2 measurement of $[Ca^{2+}]_c$

5

$[Ca^{2+}]_c$ was measured by fluorescent indicator fura-2 acetoxymethyl ester (fura-2 AM) as described in Malgaroli A et al., J. Cell. Biol. 105:2145-2155, 1987. Briefly, DU145 cell line (1×10^6 cells in triplicate culture plates) was exposed to 0, 20 and 40 mM of D-allose for 48 and 72 h and then incubated with serum-free culture media containing 5 μ M of fura-2AM at 37°C in dark for another 1 h. The cells were washed twice with Locke's solution (pH 7.3) and the fura-2 fluorescence signals of $[Ca^{2+}]_c$ were measured by luminescence spectrophotometer (LS 50B, Perkin Elmer, Boston, MA, USA) using 340 and 380 nm as excitation wavelengths (λ_{ex}), and 510 nm as emission wavelength (λ_{em}). The $[Ca^{2+}]_c$ concentration was derived from the ratio of the fluorescence intensities for each of the excitation wavelengths as mentioned above, and from the Grynkiewicz equation:

15

$$[Ca^{2+}]_c = K_d \times \frac{(R - R_{min})}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

20

where K_d : Dissociation constant of the fura-2- Ca^{2+} interaction to be 225 nM in the intracellular environment; R : Fluorescence ratio at 340 and 380 nm; R_{min} : Ratio with zero Ca^{2+} ; R_{max} : Ratio with saturating Ca^{2+} (using calcium chloride); S_{f2} : Fluorescence at 380 nm with zero Ca^{2+} ; and S_{b2} : Fluorescence at 380 nm with

5 saturating Ca^{2+} . Further, DU145 cell line was seeded onto chamber slides with 1×10^5 cells/ chamber in 500 μl of serum-free culture media containing 5 μM of fura-2 AM for fura-2 images of $[\text{Ca}^{2+}]_c$, with and without D-allose treatments (0 and 20 mM) for 48 h. The slides were mounted with Prolong Antifade reagent (Molecular Probes, Eugene, OR, USA), and images (JC-1, green) were acquired by use of a fluorescence microscope (Axioplan 2, Carl Zeiss, Germany).

【Industrial Applicability】

10 It is an effect of the present invention to provide the dose and time-dependent effects of D-allose on the proliferation of late stage human HRPC using DU145 cell line in vitro, which are highly tumorigenic and chemotherapy-resistant. Also, it is an effect of the present invention to provide the mechanism of D-allose induced apoptosis in DU145 cell line by modulation of pro- and anti-apoptotic Bcl-2 family members, Bax and Bcl-2, in favor of execution of
15 programmed cell death, which is accompanied by mitochondrial cyt C release along with concomitant alteration of mitochondrial $\Delta\psi_m$, elevation of $[\text{Ca}^{2+}]_c$, and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP).

【CLAIMS】**[Claim 1]**

A composition for inducing apoptosis in prostate cancer cells, comprising D-allose as an active ingredient.

5 **[Claim 2]**

The composition according to claim 1, wherein said prostate cancer cells are human hormone refractory prostate cancer cells.

[Claim 3]

10 The composition according to claim 1, wherein said D-allose induces apoptosis in prostate cancer cells by i) lowering the mitochondrial transmembrane potential ($\Delta\psi_m$); ii) elevating the calcium concentration in cytosol ($[Ca^{2+}]_c$); iii) cleaving the caspase 3 and poly (ADP-ribose) polymerase (PARP); and iv) releasing the cytochrome C (cyt C).

[Claim 4]

15 The composition according to claim 1, wherein said D-allose induces apoptosis by modulating expression at the transcription level of apoptosis-related genes through increasing expression of Bax and decreasing expression of Bcl-2.

[Claim 5]

20 A method for inducing apoptosis in prostate cancer cells, comprising using the composition of claim 1.

[Claim 6]

The method according to claim 5, wherein said composition is administered at 20~40 mM per 1×10^5 cells of prostate cancer cells.

[Claim 7]

A pharmaceutical composition for the prevention or treatment of prostate cancer by inducing apoptosis and inhibiting cell growth of prostate cancer cells, comprising D-allose as an active ingredient.

[Claim 8]

5 The pharmaceutical composition according to claim 7, wherein said prostate cancer is hormone refractory prostate cancer.

[Claim 9]

A composition for health functional food by inducing apoptosis in prostate cancer cells to prevent and improve prostate cancer, comprising D-allose
10 and sitologically acceptable food additive.

[Claim 10]

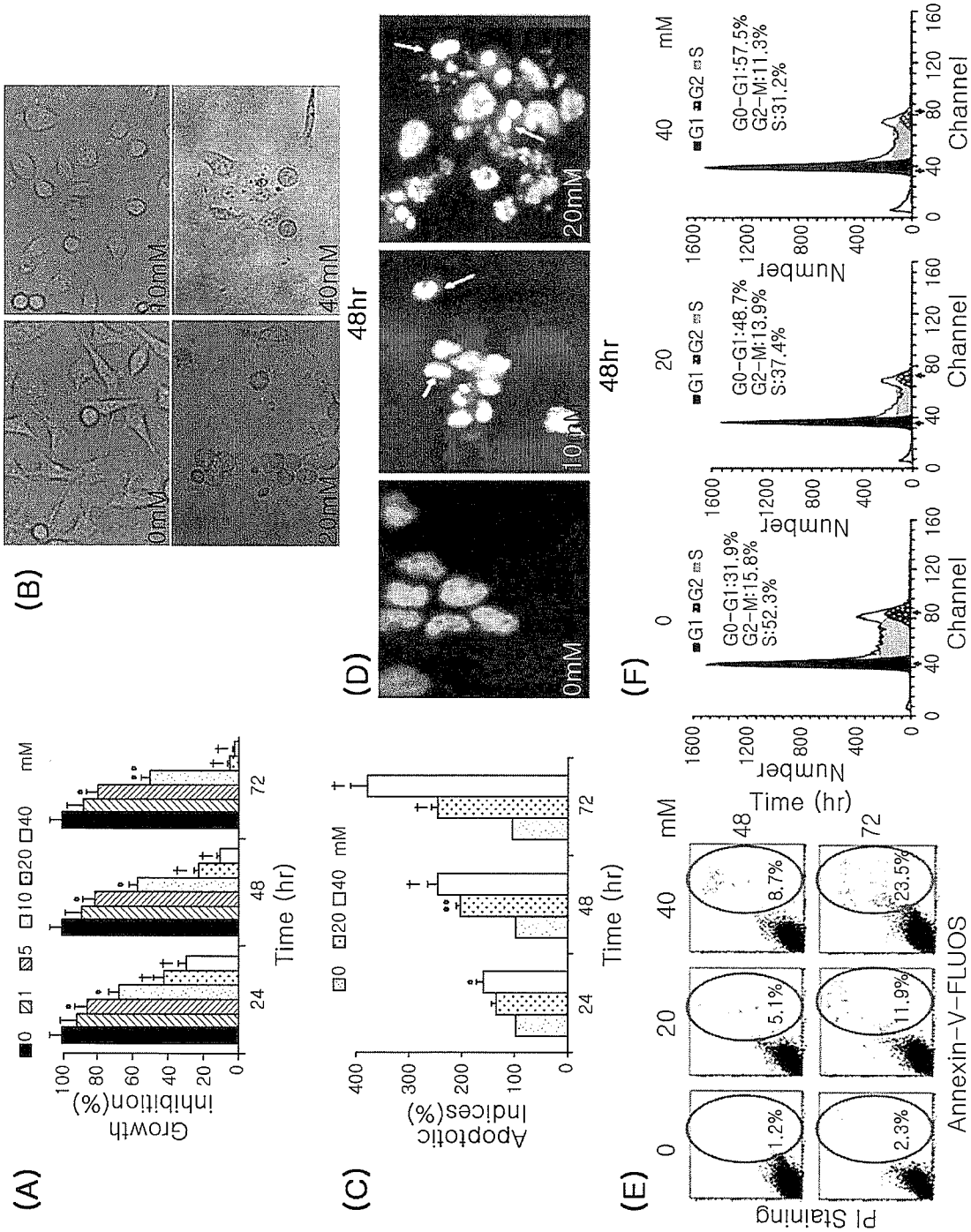
A method for treating prostate cancer by inducing apoptosis and inhibiting cell growth of prostate cancer cells, comprising administering D-allose to the individual having prostate cancer.

15 **[Claim 11]**

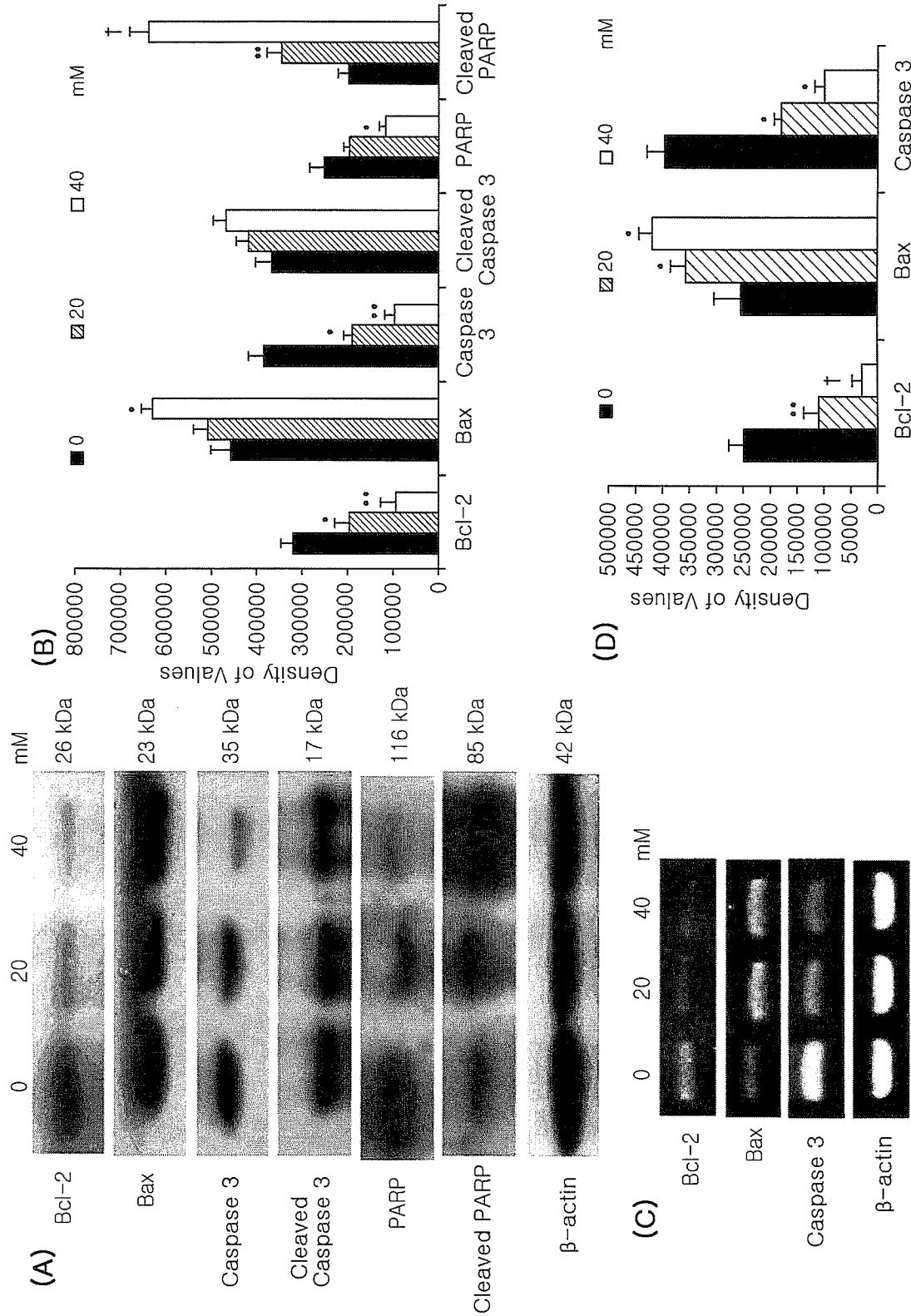
The method according to claim 10, wherein said prostate cancer is hormone refractory prostate cancer.

【DRAWINGS】

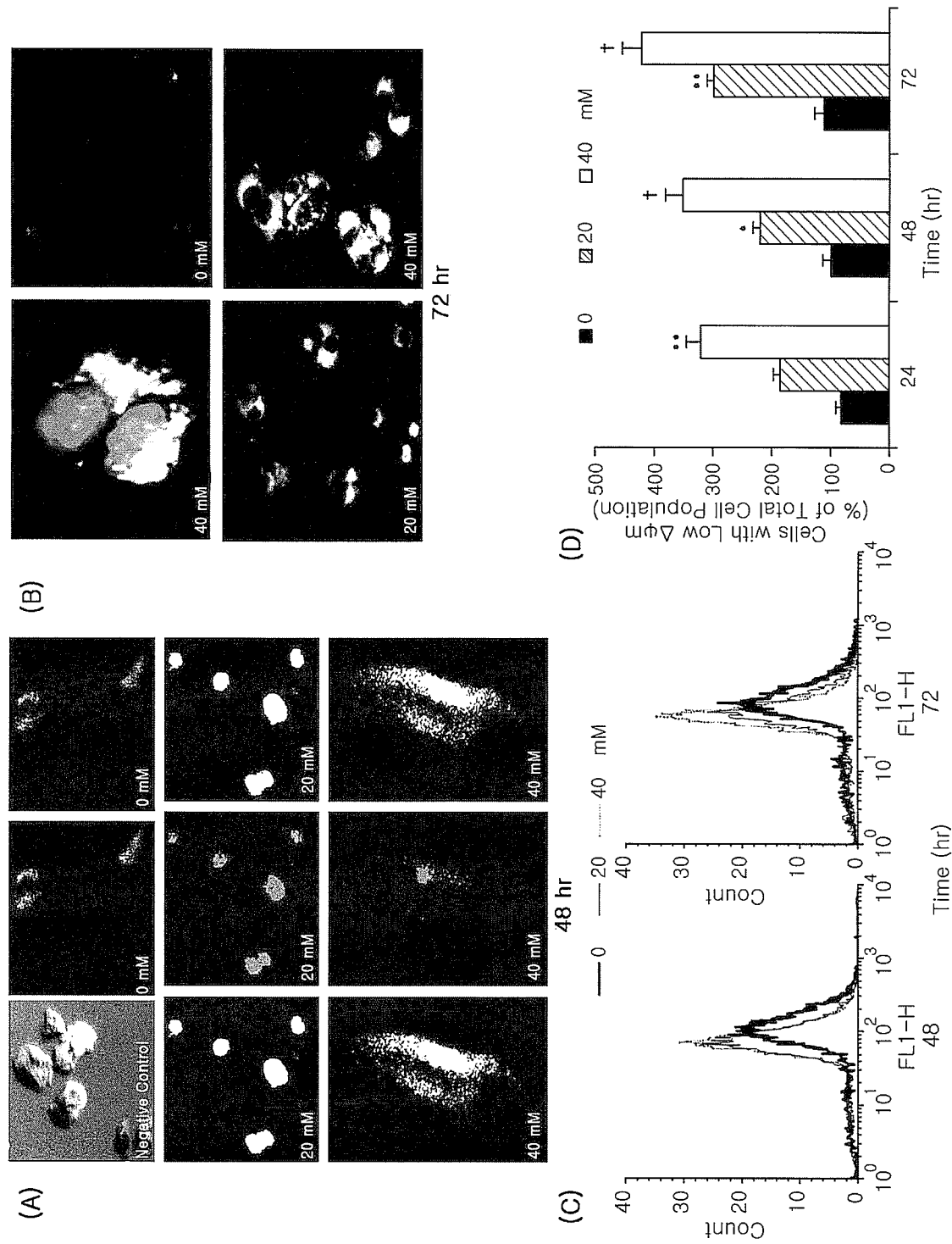
[FIG. 1]



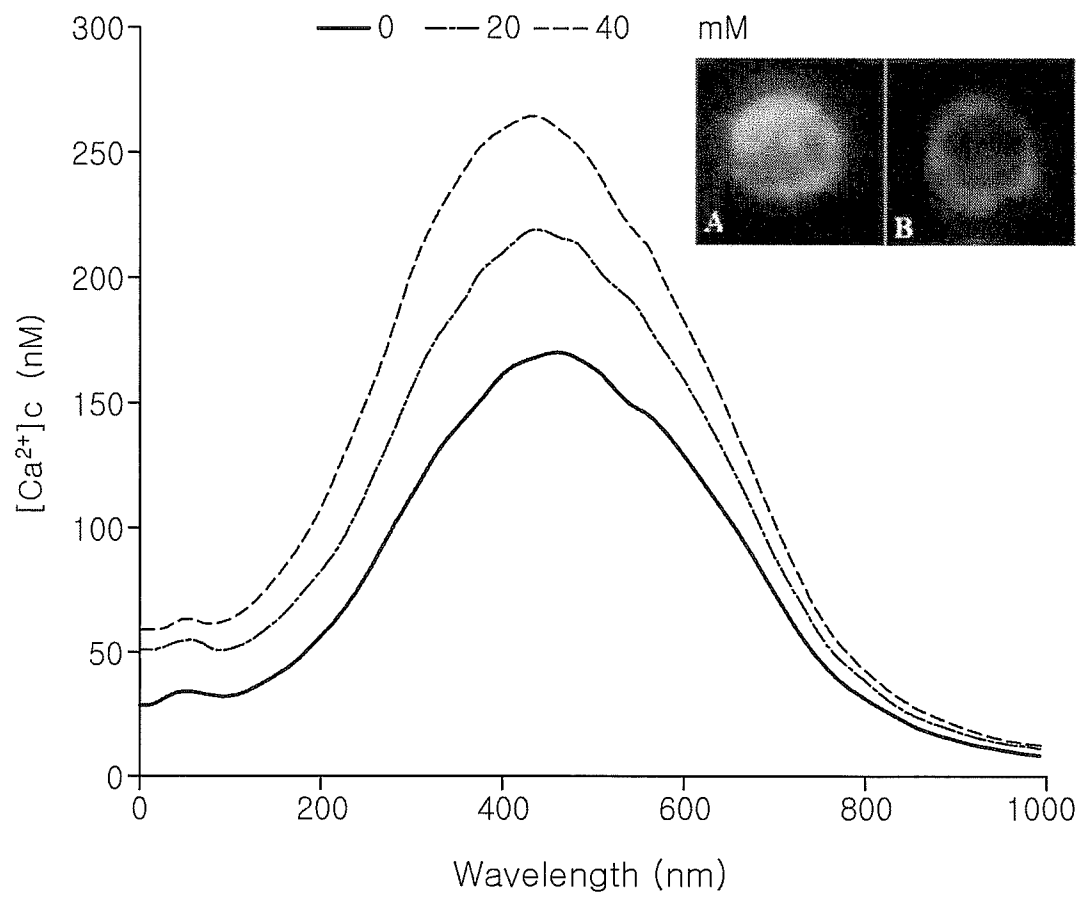
[FIG. 2]



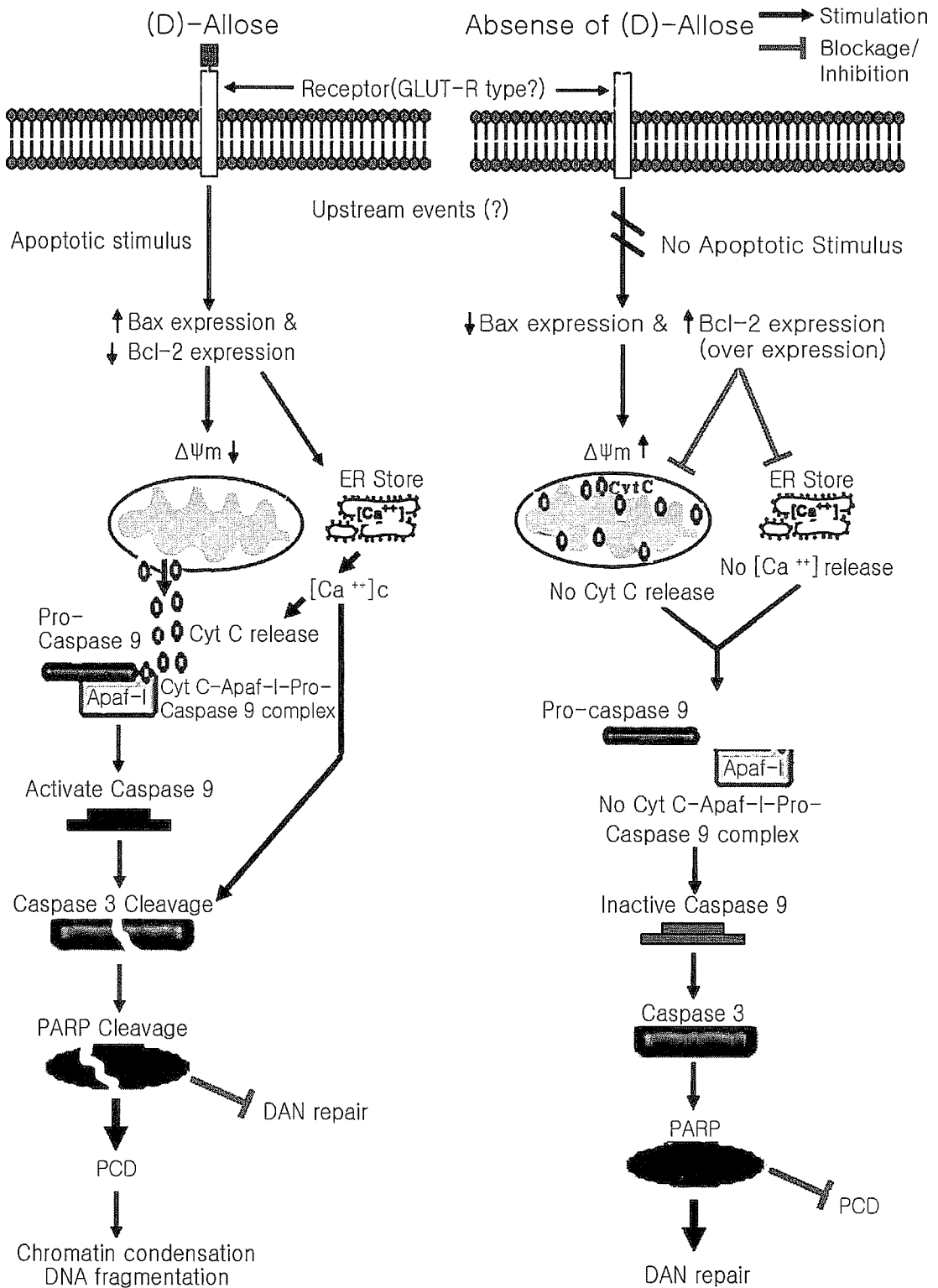
[FIG. 3]



[FIG. 4]



[FIG. 5]



A. CLASSIFICATION OF SUBJECT MATTER**A61K 31/70(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)

IPC A61K 31/70

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

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

PubMed, CA, Delphion "(D-allose) AND (cancer OR tumor)"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sui, L. et al. "The Inhibitory Effect and Possible Mechanism of D-allose on Cancer Cell Proliferation." International J. Oncology. Oct. 2005, Vol.27, No.4, Pages 907-912.	1-4, 7-9
X	Sui, L. et al. "Growth Inhibitory Effect of D-allose on Human Ovarian Carcinoma Cells in vitro." Anticancer Research. Jul. 2005, Vol.25, No.4, Pages 2639-2644.	1-4, 7-9
A	Yamaguchi, F. et al. "Rare Sugar D-allose Induces Specific Up-Regulation of TXNIP and subsequent G1 Cell Cycle Arrest in Hepatocellular Carcinoma Cells by Stabilization of p27kip1." International J. Oncology. Feb. 2008, Vol.32, No.2, Pages 377-385.	1-4, 7-9
A	US 2005/0245459 A (Fushimi Pharmaceutical Co. Ltd.) 3 Nov. 2005 See the whole document	1-4, 7-9
P, X	Naha, N. et al. "Rare Sugar D-allose Induces Programmed Cell Death in Hormone Refractory Prostate Cancer Cells." Apoptosis. Sep. 2008, Vol.13, No.9, Pages 1121-1134.	1-4, 7-9

☐ 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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 JULY 2009 (02.07.2009)

Date of mailing of the international search report

02 JULY 2009 (02.07.2009)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHANG, Je Hwan

Telephone No. 82-42-481-5634



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/007320

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/007320**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5, 6, 10, 11
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 5, 6, 10 and 11 pertains to methods for treatment of the human body by therapy, thus relates to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/007320Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 2005-0245459 A1

03.11.2005

EP 1538200 A4

06.06.2007

KR 10-2006-0071825

27.06.2006

KR 10-2008-0053407

12.06.2008