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(54) Title: METHODS FOR TREATMENT AND/OR PREVENTION OF CANCER WITH ANDROGRAPHOLIDE (AND) AND USE THEREOF

(57) Abstract: Disclosed are methods for the treatment and/or prevention of cancer such as a liver cancer or a hepatocellular carcinoma (HCC) in a subject comprising administering a therapeutically effective amount of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier and use thereof. Disclosed also are methods for preventing, treating and/or reducing liver damage in a subject comprising administering the subject a therapeutically effective amount of AND or a composition comprising AND and a pharmaceutically acceptable carrier and use thereof.



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METHODS FOR TREATMENT AND/OR PREVENTION OF CANCER WITH ANDROGRAPHOLIDE (AND) AND USE THEREOF

TECHNOLOGICAL FIELD

The present invention relates to the treatment and/or prevention of cancer in a subject. In particular the invention is directed to a composition comprising an active component for the management of cancer and use thereof.

BACKGROUND OF INVENTION

Andrographolide (AND), the active component extracted from *Andrographis paniculata* (AP), was reported to possess pharmacological properties. These include protozoacidal, anti-mutagenic, analgesic, antipyretic, and antiulcerogenic activities (82-85). The beneficial effects of AND on liver, cancer, liver cancer, liver damage or preneoplastic lesion in hepatocarcinogenesis, however, are not fully understood.

SUMMARY OF THE INVENTION

One aspect disclosed herein is directed to a method for the inhibition of proliferation of cancer cells comprising contacting the cancer cells with an effective amount of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier.

Another aspect disclosed herein is directed to a method for inducing apoptosis of cancer cells comprising contacting the cancer cells with an effective amount of AND or a composition comprising AND and a pharmaceutically acceptable carrier.

Another aspect disclosed herein is directed to a method for preventing, treating and/or reducing liver damage in a subject comprising administering the subject a therapeutically effective amount of AND or a composition comprising AND and a pharmaceutically acceptable carrier.

Another aspect disclosed herein is directed to a method for the prevention and/or treatment of liver cancer in a subject comprising administering the subject a therapeutically effective amount of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier.

Another aspect disclosed herein is directed to use of AND or a composition comprising AND and a pharmaceutically acceptable carrier for the inhibition of proliferation of cancer cells, the induction of apoptosis of cancer cells, the prevention,

treatment and/or reduction of liver damage, the prevention and/or treatment of liver cancer in a subject.

Still another aspect disclosed herein is directed to use of AND or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for the inhibition of proliferation of cancer cells in a subject.

Still another aspect disclosed herein is directed to use of AND or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for inducing apoptosis of cancer cells in a subject.

Still another aspect disclosed herein is directed to use of AND or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for preventing, treating and/or reducing liver damage in a subject.

Still another aspect disclosed herein is directed to use of AND or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for the prevention and/or treatment of liver cancer in a subject.

In an embodiment, the cancer cells are liver cancer cells, preferably human liver cancer cells.

In another embodiment, the inhibition of proliferation of cancer cells, preferably human liver cancer cells, comprises an accumulation of the cells in G₀/G₁ phase of the cell cycle.

In some embodiments, the AND or composition thereof down-regulates the expression of Bcl-2 or Bcl-w protein and/or up-regulates the expression of Bax protein in cancer cells, preferably human liver cancer cells. In other embodiments of the invention, the AND or the composition decreases the expression of Mdm2 protein and/or increases the stability of p53 protein in cancer cells, preferably human liver cancer cells.

In some embodiments, said liver cancer comprises a mammal liver cancer, preferably a human liver cancer. In other embodiment, the live cancer comprises a hepatocellular carcinoma (HCC), a cholangiocarcinoma, or a cholangiocellular carcinoma in a subject.

In some embodiments, the liver damage is induced by an agent selected from the group consisting of DEN and CCl₄. Also in other embodiments, the liver damage comprises a preneoplastic lesion in hepatocarcinogenesis. In the other embodiments of the invention, the liver damage is induced by nitrate, aflatoxin metabolites or phenobarbital. Still in some embodiments of the invention, said preventing, treating and/or reducing liver damage comprises restoring the morphology of normal liver.

In some embodiments, each of the methods disclosed herein can be performed *in vitro*, *ex vivo*, or *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

As shown in Figure 1, HepG2 cells treated with different concentrations of AND are incubated for 24, 48 and 72 hrs. Neutral red assays are performed and absorbance is read at OD 540 nm. IC₅₀, the concentration of AND that reduces the cell viability by 50%, is recorded at different incubation time periods.

As shown in Figure 2, the same treatments as those in Fig. 1 are performed with WRL 68, the human normal liver embryo cells. Neutral red assays are performed and IC₅₀ is determined for each incubation time period.

As shown in Figure 3, Similar treatments to those in Figures 1 and 2 are performed with Clone 9, the rat normal liver cells. Neutral red assays are performed and IC₅₀ is recorded for each incubation time period.

Figure 4A-C show DNA profiles of HepG2 cells treated with DMSO and AND for 24 hrs. HepG2 cells incubated with (A) 1% DMSO, (B) 20 μ M and (C) 25 μ M of AND for 24 hrs were stained with PI. The DNA profiles were obtained using FACScan flow cytometer and analyzed using FCS Express (V2) software. The area of the profile (in red) represents the total number of cells counted.

Figure 5 shows that after PI staining, 10,000 HepG2 cells are counted on FACScan flow cytometer and the number of cells suspended in each phase of the cell cycle (sub G₁, G₀/G₁, S and G₂/M) is represented in percentage. The data represent mean $\pm$ SD ($n = 2$).

Figure 6A-C shows that after PI staining, the DNA profiles of HepG2 cells incubated with (A) 1% DMSO, (B) 12.5 μ M and (C) 25 μ M of AND for 48 hrs are obtained using FACScan flow cytometer and analyzed using FCS Express (V2) software.

Figure 7 shows analysis of cell cycle upon treatment of DMSO and AND for 48 hrs. HepG2 cells (10,000) are counted on FACScan flow cytometer after PI staining. The number of cells suspended in sub G₁, G₀/G₁, S and G₂/M is recorded and represented in percentage. All data are expressed as mean $\pm$ SD ($n = 2$). Significance is found between control and the cells treated with 12.5 or 25 μ M AND in all phases of cell cycle ($p < 0.05$).

Figure 8 shows that at 72 hrs after treatment of HepG2 cells with DMSO or 12.5, 20, 25 and 50 μ M of AND, DNA is extracted. Fragmented DNA is observed in cells incubated with 50 μ M of AND.

Figure 9 shows the scatter plot of gene expressions of HepG2 cells treated with DMSO and AND. Each symbol represented one gene. The center line indicates no changes in gene expression. Parallel lines represent the boundary which was set to be 3. The black symbols indicate the genes of which expression changes were less than the boundary. Red symbols indicate an increase in gene expression from X axis to Y axis greater than the boundary. Green symbols indicate a decrease in gene expression from X axis to Y axis greater than the boundary.

Figure 10A-B show cDNA microarray analysis of (A) DMSO control and (B) AND-treated cells. (C) Upon treatment of 16 μ M of AND for 48 hrs, six genes are up-regulated 3-folds or more than the control; forty-two genes are down-regulated 3-folds or more and some of them are listed above.

Figure 11A shows the rat is treated with DEN & CCl₄ once a week for about 5 months. Figure 11B shows the rat's liver is removed and many nodules as indicated by the arrows is observed across the surface of the liver.

Figure 12A-C show that after 28 weeks, rats are killed and their livers are perfused, removed & weighed. Liver removed from (A) a normal control rat; (B) AND-treated rat and (C) positive control rat. Nodules or lumps as indicated by the arrows can be seen across the surface of the positive control liver.

Figure 13A-C show that at the end of 56th week, rats are killed and their livers are examined. Panel A shows negative control liver; panel B shows AND-treated liver and panel C shows positive control liver.

Figure 14 A-B show that the relative liver weight is expressed as percentage (liver weight/ body weight). The data represent the mean of 5 rats. There is a significant difference between positive control and the AND-treated groups in both the promotion (A) and progression (B) ($P < 0.05$).

Figure 15A-B show AST and ALT assays for the (A) promotion and (B) progression groups of rats. The amount of AST and ALT in blood serum is measured and represent the percentage compared with the negative control. Significance is found between the positive control and AND-treated rats in both the promotion and progression groups ($P < 0.05$).

Figure 16A-D show the nuclei of the hepatocytes are stained blue and the cytoplasm is stained red. Panel A shows the liver section (10X) of a negative control rat. Panel B shows magnification of the square area in section A (20X). Panel C shows positive control liver sections (20X). Panel D shows AND-treated liver section (20X).

Figure 17A-D show A) The liver section of a negative control rat (20X); B) The liver section of a rat treated with AND (20X); C) Positive control sections with no distinct cytoplasm (10X); and D) hepatocytes were arranged randomly across the whole section (20X).

Figure 18A-F show that the nuclei of the hepatocytes are stained blue and the areas with GST-P expression are stained brown. Panel A shows showed no expression of GST-P in negative control section (10X). Panel B shows magnification of the section in A (20X). Panel C shows liver section from a positive control rat (10X). Panel D shows magnification of the section in C (20X). Panel E shows liver section obtained from AND-treated liver (10X). Panel F shows the magnified area from section E (20X).

Figure 19A shows the negative control sections with distinct central vein (20X). Figure 19B shows expressions of GST-P in positive control section in groups (10X). Figure 19C shows the appearance of hepatocytes in positive control section (20X). Figure 19D shows positive control section with no GST-P expression (20X). Figure 19E shows the AND-treated liver sections (10X). Figure 19F shows the magnified area in section E (20X).

Figure 20A-B show that the cytosolic proteins in the rat liver are separated by 10% SDS-PAGE and transferred onto PVDF membrane. Panel A shows that PCNA is detected and expressed in PCNA : β -actin ratio. Significance is found between the AND-treated and positive control groups in the progression experiment ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Panel B shows PCNA expression. β -actin is the internal standard.

Figure 21A-B show that the cytosolic protein, Bax, in the rat liver is separated by 10% SDS-PAGE and transferred onto PVDF membrane. It is detected with mouse antibodies against Bax followed by goat anti-mouse-HRP visualization. As shown in panel A, Bax is expressed in Bax : β -actin ratio. Significance is found between the AND-treated and positive control groups in both experiments ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Penal B shows Bax expression. β -actin is the internal standard.

Figure 22A-B show that the cytosolic proteins in the rat liver are separated by 10% SDS-PAGE and transferred onto PVDF membrane. Bcl-2 is detected with mouse antibodies against Bcl- 2 followed by goat anti-mouse-HRP visualization. As shown in panel A, Bcl-2 is expressed in Bcl-2 : β - actin ratio. Significance is found between the AND-treated and positive control groups in both experiments ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Penal B shows Bcl-2 expression. β -actin is the internal

standard.

Figure 23A-B show that the protein p21 in the rat liver is separated by 10% SDS-PAGE and transferred onto PVDF membrane. It is detected with mouse antibodies against p21 followed by goat anti-mouse-HRP visualization. As shown in panel A, p21 was expressed in p21 : β -actin ratio. Levels of p21 in AND-treated groups are higher than positive control groups, but no significant difference is observed in both experiments. All data are expressed as mean $\pm$ SD ($n = 5$). Panel B shows p21 expression. β -actin is the internal standard.

Figure 24 A-B show that total p53 is separated by 10% SDS-PAGE and then transferred onto PVDF membrane. The nuclear protein is detected with mouse antibodies against p53 (Pab 421) and goat anti-mouse-HRP. As shown in panel A, total p53 is expressed in p53 : β -actin ratio. There is no significant difference in the levels of total p53 in AND-treated groups as compared with the positive control groups in both experiments. All data are expressed as mean $\pm$ SD ($n = 5$). Panel B shows total p53 expression. β -actin is the internal standard.

Figure 25A-B show that nuclear proteins are immunoprecipitated with mouse monoclonal anti-p53 antibody (Pab 246). The precipitated protein are separated and transferred onto the PVDF membrane. Wildtype p53 is detected with mouse antibodies against wt p53 (Pab 421) and goat anti-mouse-HRP. As shown in panel A, wt p53 is expressed in wt. p53 : β -actin ratio. There is no significant difference in the levels of wt. p53 in AND-treated group as compared with that in the positive control group in the promotion experiment. In the progression experiment, however, a significant increase in the level of wt. p53 in AND-treated group is observed when compared with that in the positive control group ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Panel B shows wt. p53 expression. β -actin is the internal standard.

Figure 26 A-B show that the nuclear proteins in the rat liver are separated by 10% SDS-PAGE and transferred onto PVDF membrane. Mdm2 is detected with mouse antibodies against Mdm2 and goat anti-mouse-HRP. As shown in panel A, Mdm2 is expressed in Mdm2 : β -actin ratio. There is a significant decrease in the levels of Mdm2 in AND-treated groups as compared with the positive control groups in both experiments ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Panel B shows Mdm2 expression. β -actin is the internal standard.

As shown in Figure 27A, p53 expression is measured as the ratio of p53 : β -actin. There is a significant reduction in the expression in the AND-treated group when

compared to that in the positive control group in both experiments ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Figure 27B shows B *p53* mRNA expression. β -actin is the internal standard.

As shown in Figure 28A, *Mdm2* expression is measured as the ratio of *Mdm2* : *β -actin*. There is a significant reduction in the expression in the AND-treated group when compared to that in the positive control group in both experiments ($p < 0.05$). All data are expressed as mean $\pm$ SD ($n = 5$). Figure 28B shows *Mdm2* mRNA expression. β -actin is the internal standard.

Figure 29 shows the treatment schedule of the rats in the promotion stage of hepatocarcinogenesis. Group I represents the negative control rats which did not receive any toxicants. Group II is the positive control rats which were exposed to DEN and CCl₄. Group III represents the AND-treated group which was not given treatment until the 5th week of the experimental schedule.

Figure 30 shows the schedule of the rat treatments in the progression stage of hepatocarcinogenesis. Group I represents the negative control rats which did not receive any toxicants. Group II is the positive control rats which were exposed to DEN and CCl₄. Group III represents the AND-treated group. Oral treatments for the three groups were not given until the 32nd week of the experimental schedule.

DETAILED DESCRIPTION OF THE INVENTION

The term “therapeutically effective amount” or “effective amount” used herein is intended to mean an amount of the active component effective to achieve its intended purposes, such as inhibition of cancer cell proliferation, induction of cancer cell apoptosis, prevention, treatment and/or reduction of liver damage, prevention and/or treatment of liver cancer in a subject. The amount or dose will vary depending upon the symptoms, sex, age, and weight of patients, method of administration, time and intervals of administration and properties, dispensing, and kind of pharmaceutical formulations, specific effective ingredients, etc. Normally the active components such as AND may be administered in a dose of about 0.001 to 1,000mg, preferably 0.01 to 100mg, more preferably 0.1 to 50 mg, most preferably 1 to 20mg, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20mg, per day each kilogram of body weight per subject, administered in single or divided doses, administered in single or divided doses. In some cases, however, it may be necessary to use dosages outside these limits, which will be determined by the prescribing physician.

The term “treating” or “treatment” used herein includes abrogating, substantially inhibiting, slowing or reversing the progression of a disease or condition, ameliorating symptoms of the disease or condition, such as cancer, or liver damage. The term “preventing” or “prevention” used herein includes preventing occurrence of a disease or condition, or the appearance of clinical, functional or aesthetic systems of the disease or condition, such as cancer, or liver damage.

The term “pharmaceutically acceptable carrier” used herein means excipients and auxiliaries which facilitate processing of the active component into formulations which can be used pharmaceutically. The formulations can be administered orally, intramuscularly, intraperitoneally, subcutaneously and intravenously. Preferably, the formulations, particularly those such as tablets, dragees, troches and capsules, as well as suitable solutions, contain from about 0.01 to 99.99 percent by weight, preferably from about 25 to 75 percent by weight of active component(s) such as ANP of the invention together with the excipient and/or auxiliary. In some embodiments, the formulation contains one, a half, one third, one fourth, or one fifth of the above mentioned dose of ANP for the subject to be administered.

Suitable excipients used as carriers includes fillers such as saccharides, for example, lactose or sucrose, mannitol or sorbitol; cellulose derivatives; magnesium sulfate; calcium phosphates such as tricalcium phosphate or calcium hydrogen phosphate; as well as binder such as starch paste, for example, maize starch, wheat starch, rice starch, potato starch; gelatin; tragacanth; and/or polyvinylpyrrolidone.

Suitable auxiliaries that may be used as a carrier include flow-regulating agents and lubricants, such as talc, silica, stearic acid or salts thereof (such as magnesium stearate), and/or polyethylene glycol. Dyestuffs or pigments can be added to the tablets or dragee coatings.

The composition disclosed herein may be formulated in the form of injections, such as intravenous, subcutaneous, and intramuscular injections, suppositories, or sublingual tablets. Pharmaceutical formulations in the dosage form of, e.g., injections, suppositories, sublingual tablets, tablets, and capsules are prepared according to methods which are commonly accepted in the art.

In preparing injections, the effective ingredient is blended, if necessary, with a pH modifier, a buffer, a solubilizing agent, a suspending agent, a stabilizer, and a preservative, followed by preparation of an intravenous, subcutaneous, or intramuscular injection according to an ordinary method.

Examples of the solubilizing agent include polyoxyethylene hydrogenated castor oil, polysorbate 80, nicotinamide, polyoxyethylene sorbitan monolaurate, macrogol, and an ethyl ester of castor oil fatty acid. Examples of the suspending agents include methylcellulose, polysorbate 80, hydroxyethylcellulose, acacia, powdered tragacanth, sodium carboxymethylcellulose, and polyoxyethylene sorbitan monolaurate.

Examples of the stabilizer include sodium sulfite, sodium metasilicate, and ether, and examples of the preservative include methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, sorbic acid, phenol, cresol, and chlorocresol.

In an embodiment of the invention, when the active compound or the composition is administered orally, it can be in the form of tablets or capsules, or as an aqueous solution or suspension. In the case of tablets, carriers which are commonly used include lactose, mannitol and corn starch, and lubricating agents, such as magnesium stearate, are commonly added. In the case of the capsule form, the active components can be administered in dry form in a hard gelatin capsule or in a suitable gelled or liquid vehicle, such as a liquid polyethylene glycol or a carrageenan gel, in a soft gelatin capsule. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents may be added.

In a preferred embodiment, the pharmaceutical formulation in the dosage form of, an aqueous solution or suspension for injection or oral administration comprises a oil solution of AND, more preferably a corn oil solution thereof.

As used herein, the terms "subject" and "subjects" refers to an animal (e.g., birds, reptiles, and mammals), preferably a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, cat, dog, rat, and mouse) and a primate (e.g., a monkey, chimpanzee, and a human).

The following examples are intended to illustrate embodiments of the invention, and should not be construed as limitations on the scope of the invention.

EXAMPLES

Materials & Methods -- *In vitro*

In this part of the experiment, the biological activity of andrographolide in liver cancer cell line was investigated. Neutral red assay, cell cycle analysis, DNA fragmentation, and cDNA microarray were employed to study the effects of the active

component on HepG2 cells.

EXAMPLE 1: Effects of andrographolide on cell viability and cell cycle

Neutral red assay, flow cytometry & DNA fragmentation

1.1 Materials & Solutions

Table 1. Materials & solutions as used

Trypsin-EDTA (1X), Dulbecco's Modified Eagle Medium (DMEM), RPMI Medium 1640, PSN Antibiotic mixture	Invitrogen (Ca., USA)
Fetal Bovine Serum (FBS)	Biosera (UK)
Sodium chloride (NaCl), potassium chloride (KCl), sodium dodecyl sulfate (SDS), agarose, proteinase K, bromophenol blue	USB (Cleveland, USA)
Sodium phosphate (NaH_2PO_4), potassium (KH_2PO_4), neutral red (NR), sodium bicarbonate (NaHCO_3), propidium iodide (PI), ethylenediaminetetraacetic acid (EDTA), xylene cyanole, ethidium bromide (EB)	Sigma Chemicals (St. Louis, USA)
Andrographolide (AND) 98%	Sigma-Aldrich (USA)
Dimethylsulfoxide (DMSO)	Fisher Scientific (USA)
DNA ladder (GeneRuler)	Fermentas (Ont. Canada)
RNase A	Amersham (USA)
Steritop-GP 500 ml (0.22 μm), millex-GP (0.22 μm)	Millipore (Ma., USA)
75 cm^2 culture flask, 96-well microplate	Iwaki (Tokyo, Japan)
60 mm culture dish	Corning (USA)
Flow cytometer	Beckman Coulter (USA)

1.2 Preparation of solutions

RPMI: The medium was prepared by dissolving one pack of RPMI medium and 2.0 g of NaHCO_3 in 895 ml of dH_2O . After the powder was completely dissolved, the solution was adjusted to pH 7.2. The solution was sterilized in the culture hood by filtering through a Steritop membrane with pore sizes of 0.22 μm . Fetal bovine serum (100 ml) and PSN antibiotic mixture (2 ml) were added to the filtered solution and the completed medium was stored at 4°C. All containers for cell culture use were sterilized.

DMEM: The medium was prepared by dissolving one pack of DMEM medium

and 3.7 g of NaHCO_3 in 895 ml of dH_2O . After pH was adjusted to 7.2, membrane filtration was performed in the culture hood. Before use, 100 ml of FBS and 2 ml of PSN antibiotic mixture were added to the filtered solution and the completed medium was stored at 4°C . The containers for cell culture use were sterilized.

10 X Phosphate-buffered saline (PBS): The solution contained 80 g of NaCl, 2 g of KCl, 14.4 g of NaH_2PO_4 , and 2.4 g of KH_2PO_4 in 900 ml of dH_2O . The pH of the solution was adjusted to 7.4, and the volume was made up to 1000 ml with dH_2O .

1 X PBS: It was made by diluting 100 ml of 10 X PBS with 900 ml of dH_2O . This solution was autoclaved for cell culture use.

Andrographolide (AND) stock solution (20mM): It was made by dissolving 7.6 mg of AND in 1.08 ml of DMSO. Different concentrations of AND were prepared by diluting the stock solution with culture medium.

NR solution: The solution was made by dissolving 1 g of NR in 200 ml of 1 X PBS, followed by membrane filtration using Millex GP with pore size of $0.22\ \mu\text{m}$.

Lysis buffer: It contained 200 mM Tris-HCl, pH 8.3, 100 mM EDTA and 1% SDS.

Proteinase K solution (10 mg/ml): The solution was made by dissolving 5 mg of proteinase K in 0.5 ml of dH_2O .

6 X DNA loading dye: It was made up of $93.6\ \mu\text{l}$ of glycerol, $3\ \mu\text{l}$ of 0.5 M EDTA (pH 8.0), 0.3 mg of bromophenol blue, 0.3 mg of xylene cyanole and dH_2O to a final volume of $250\ \mu\text{l}$.

10 X Tris-Borate-EDTA (TBE) buffer: The buffer was prepared by dissolving 108 g of Tris base, 55 g of Boric acid and 9.3 g of Na_4EDTA in 1000 ml of distilled water.

Ethidium bromide (EB): It was prepared by dissolving 50 mg of EB in 100 ml of dH_2O .

1.3 Procedures

1.3.1 Seeding cells into culture flask (performed inside culture hood)

Cells stored in liquid nitrogen were thawed in a 37°C water bath, along with 1 X PBS and completed medium. Cells were transferred to a new tube and were centrifuged at 1,000 rpm for 3 min. The supernatant was discarded and the cell pellet was washed twice with PBS by centrifugation at 1,000 rpm for 3 min. The supernatant was discarded and 1 ml of the completed medium was added to resuspend cells. After that, cells were transferred to a new $75\ \text{cm}^2$ culture flask with 12 to 15 ml of warm medium. The flask was

stored in a 37°C incubator supplied with 5% CO₂.

1.3.2 Subculturing technique (performed inside culture hood)

The medium was discarded from the 75 cm² culture flask and cells were rinsed with warm PBS twice to remove any trace of serum. Trypsin-EDTA (1 ml) was added to the flask and incubated at 37°C. After a few minutes, four milliliters of completed medium were added to stop the activity of trypsin. The cells were transferred to a new tube and spinned at 1,000 rpm for 3 min. The supernatant was discarded and the cell pellet was resuspended in warm medium. Cells were washed again by centrifugation at 1000 rpm for 3 min, and were resuspended in 1 ml medium. Subsequently, 2 µl of cells was added to 18 µl of dye and the mixture was placed onto a hematology analyzer for cell counting. Approximately 0.5 ml of 1×10^6 cells was seeded in a new 75 cm² culture flask with 12 ml of fresh medium. The culture flask was put inside a 37°C incubator with 5% CO₂.

1.3.3 Neutral red assay (cell viability test for HepG2, WRL 68 & Clone 9 cells)

The inhibitory effect of AND on cell viability was measured by neutral red assay. HepG2, the human liver cancer cells, were thawed from stock using RPMI medium. After several subcultures, 100 µl of 1×10^4 cells was seeded onto 96-well plates and allowed to be pre-treated for 24 hrs. After that, attached cells were incubated with 100 µl of 1% DMSO as the control or different concentrations of AND. The plates were incubated at various time periods: 24, 48 and 72 hrs. After treatment, the culture medium was removed and cells were washed with 200 µl of non-sterilized PBS twice. Then, neutral red solution (50 µl) was added to all the wells except the blank. The plates were wrapped in aluminum foil and incubated for 1 hr at 37°C. After that, wells were washed with 200 µl of PBS twice and the plates were inverted and allowed to dry in a 65°C oven. To each well of the plates, 100 µl of 1% SDS was added. The plates were shaken for 2 min and placed in a microplate reader to measure the absorbance at 540 nm. The cell viability (%) was plotted against the concentration of AND and the IC₅₀, the concentration of the agent that reduced the cell viability by 50%, was recorded.

The same procedure was applied to human normal liver embryo cells, WRL 68, and rat normal liver cells, Clone 9, except that the culture medium was DMEM for these two cell lines.

1.3.4 DNA purification of HepG2 cells

Apoptotic cells were detected by DNA fragmentation. Approximately 1×10^6 HepG2 cells were seeded onto a 60 mm culture dish and incubated with 1% DMSO or different concentrations of AND (12.5, 20, 25 and 50 μM). After 72 hrs, cells were harvested and washed with PBS by centrifugation at 1,000 for 3 min. The cell pellet was resuspended in 400 μl of lysis buffer in a 1.5 ml microtube with gentle vortexing. When no cell debris was left, 20 μl of 10 mg/ml of proteinase K was added to each tube and the lysed cells were incubated at 37°C for 3 hrs. After samples were cooled to room temperature, 150 μl of saturated NaCl solution was added and the contents were mixed by vigorous shaking. The tubes were then centrifuged at 7,000 rpm for 15 min at room temperature. The supernatant containing DNA was poured to a new tube, and 1 ml of cold ethanol was added. After the tube was inverted several times, it was centrifuged at 14,000 rpm at 4°C for 20 min. Samples were washed once with 70% EtOH and the DNA pellet was allowed to air dry for 15 to 20 min. TE buffer (20 to 50 μl) containing 0.2 mg/ml of RNase A was added to each DNA pellet. The samples were incubated at 37°C for 90 min. About 2 μl of the DNA solution was added to 998 μl of TE buffer and concentration was measured using UV spectrophotometry (Beckman, DU 650) with OD_{260} .

1.3.5 DNA gel electrophoresis

DNA gel electrophoresis was performed to detect DNA fragmentation. DNA gel containing 1.5% (w/v) agarose and 0.5 X TBE was boiled to completely dissolve the agarose. The gel solution was mixed thoroughly and 5 $\mu\text{g/mL}$ of EB was added when the solution had cooled down to room temperature. Then the solution was poured into a gel casting mould for solidification. After the concentration of each sample was adjusted to 625 $\mu\text{g/mL}$, 10 μl of DNA samples were mixed with 2 μl of 6 X DNA loading dye and the mixture was loaded into the wells. The gel was run at 60 V for 45 min in 0.5 X TBE buffer. Electrophoresis was completed when the bromophenol blue had migrated down 2/3 of the length of the gel. The DNA bands were visualized under UV illuminator (UVP) and photographed for record.

1.3.6 Flow cytometry (cell cycle analysis)

The nuclear DNA content was measured by using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis. HepG2 cells were counted and approximately 1×10^6 cells were seeded onto a 60 mm culture dish and cells were pre-incubated for 24 hrs. After incubation with 1% DMSO or 12.5, 20 and 25 μM of AND

for 24 and 48 hrs, floating cells in the spent medium and adherent cells were collected by combining the spent medium and trypsin-treated samples. Cells were harvested by centrifugation at 1,000 rpm for 3 min. The medium was discarded and the cell pellet was washed once with 1 ml PBS by centrifugation. The cell pellet was resuspended with 0.1 ml PBS and fixed in 1 ml of ice-cold 70% ethanol. The cell suspension was allowed to store overnight at 4°C. After fixing, cells were washed once with 1 ml PBS by centrifugation at 1,000 for 3 min at room temperature and resuspended in 1 ml PBS containing 8 µg/ml of RNase A and 40 µg/ml of PI. Samples were then incubated at 37°C for 15 to 30 min and DNA content was analyzed using a FACScan flow cytometer.

The total number of cells counted was set to 10,000. The DNA profile was represented in 1D plot (histogram), where the x-axis is the channel number and y-axis the number of events. After the profiles were obtained, the data was analyzed using FCS Express (Version 2) software by De Novo Company. The histograms showing the DNA profiles of the control and AND-treated groups were compared and the number of cells suspending in Sub G₁, G₀/G₁, S and G₂/M phases were counted.

EXAMPLE 2: Effects of andrographolide on gene expressions

mRNA extraction from cells and cDNA microarray

2.1 Materials & Solutions

Table 2. Materials & solutions as used

Agarose, diethyl pyrocarbonate (DEPC), MOPS,	USB
Chloroform, EDTA, isopropanol, formaldehyde, tris, deionized formamide, ethidium bromide (EB)	Sigma
Trizol reagent	Gibco BRL
Ethanol (EtOH)	BDH
Oligo GEArray Human Toxicology & Drug metabolism microarray membrane (OHS-401), ArrayGrade cRNA cleanup kit, TrueLabelling-AMP 2.0, Oligo GEArray Reagent Kit	SuperArray, Bio-Gene Tech.
Biotin-16-UTP	Roche Applied Science

2.2 Preparation of solutions

Diethyl pyrocarbonate (DEPC)-treated water: DEPC was added to autoclaved nano-pure water to make a final concentration of 0.1% by volume. It was stirred overnight

and autoclaved before use.

1 X TE buffer: It contained 10 mM Tris-HCl and 1 mM EDTA. The pH was adjusted to 8.0 with HCl.

10 X MOPS: It was prepared by dissolving 83.72 g of MOPS in 450 ml of DEPC-treated dH₂O, and pH was adjusted to 7.0. Then 8.203 g of NaOAc and 3.722 g of EDTA were added to the solution. A final volume of 500 ml was made up with DEPC-dH₂O.

RNA sample buffer: It contained 10 ml of deionized formamide, 3.5 ml of 12.3 formaldehyde, 1 ml of 10 X MOPS buffer and 1 ml of DEPC-treated water and stored at -20°C.

6 X RNA loading dye: It was made up of 10 mM of EDTA (pH 8.0), 50% (v/v) glycerol, 0.25% bromophenol blue (w/v) filter-sterilized and stored at -20°C.

2.3 Procedures:

2.3.1 Cell treatments

HepG2 cells (5×10^6) were seeded in a culture flask for 24 hrs in a 37°C incubator. After incubation, cells were treated with 1% DMSO or 16 μ M of AND for 48 hrs. The medium was discarded and cells were trypsinized and washed with PBS twice by centrifugation at 1,000 rpm for 3 min.

2.3.2 mRNA extraction from cell

a) Cell lysis: 0.5 ml of Trizol reagent was added to the cell pellet, vortexed and stored at room temperature for 10 to 15 min. It was then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to a new tube.

b) RNA Extraction: 0.1 ml of chloroform was added to the supernatant, and mixed vigorously. The sample was stored at room temperature for 10 to 15 min. It was then centrifuged at 12,000 g for 15 min at 4°C.

c) RNA Precipitation: the aqueous layer, which contained RNA, was transferred into a new tube. Isopropanol (0.25 ml) was added and stored at room temperature for 10 min. The sample was centrifuged at 12,000 g for 15 min at 4°C.

d) RNA Wash: the RNA pellet was mixed with 0.5 ml of 75% ethanol. It was then centrifuged at 7,500 g for 5 min at 4°C.

e) Solubilization: ethanol was discarded and the RNA pellet was allowed to air dry for 15 to 30 min. It was dissolved in 50 μ l of DEPC-treated dH₂O and incubated at 55

to 60°C for 10 min.

f) Storage: It was kept at -20°C for temporary storage or at -70°C for a long term storage.

2.3.3 Determination of total RNA yield and quality yield

The RNA sample was diluted in 1:500 with 1 X TE buffer. The yield of total RNA was obtained by UV spectrophotometry (Beckman, DU 650) at wavelength 260 nm, using 1 X TE buffer as blank. The amount of total RNA was calculated by the formula that 1 A_{260} unit has 40 $\mu\text{g/ml}$ of single stranded RNA. The result was divided by 2 as the dilution factor. The quality of RNA was assessed by the ratio of A_{260}/A_{280} . The integrity of the RNA was determined by formaldehyde agarose gel electrophoresis. The ratio of 28S and 18S ribosomal RNAs was close to 2:1 after EB staining.

2.3.4 RNA formaldehyde agarose gel eletrophoresis

The agarose gel was prepared by dissolving 0.3 g of agarose in 14.4 ml of DEPC-treated water. The solution was heated in a microwave to completely dissolve the agarose. When the mixture was cooled down for a few minutes, formaldehyde and 1 X MOPS were added to the gel solution. It was mixed thoroughly, and was poured into the gel casting mould for solidification.

Table 3. Protocol for formaldehyde agarose gel

Agarose	0.3 g or 1.5% (w/v)
DEPC-treated water	14.4 ml
1 X MOPS	2 ml
Formaldehyde	3.6 ml

The RNA sample for gel electrophoresis was prepared by mixing 2 to 3 μg of RNA with 10 μl of RNA sample buffer, 1 μl of RNA loading dye and DEPC-treated water to make a final volume of 20 μl . The mixture was heated to 65°C for 10 min, and allowed to cool to room temperature before loaded into the gel.

After samples had been loaded into the wells, the gel was run in 1 X MOPS buffer at 120 V for 20 min or until the bromophenol blue dye had migrated down 2/3 of the length of the gel. The gel was stained with 5 $\mu\text{g/ml}$ of EB for 30 min and then destained with three rinses of DEPC-treated water. The RNA bands were examined under UV illuminator (UVP) and it was photographed for record.

2.3.5 cDNA synthesis

Table 4. cDNA SuperArray kit components

Tube	Contents
G1	TrueLabeling Primer
R1	RNase Inhibitor
G2	cDNA Synthesis Enzyme Mix
G3	5 X cDNA Synthesis Buffer
H ₂ O	RNase-free H ₂ O
G24	2.5 x RNA Polymerase Buffer
G25	RNA Polymerase Enzyme
Spin columns	
Elution Tubes	
GEAhyb. Hybridization solution	
Solution Q	
5X buffer F	
AP-SA	
Buffer G	
CDP-Star chemiluminescent substrate	

The components in G1, G3, H₂O, and G24 were thawed, mixed and collected at the bottom of the tube with a brief spin in a microcentrifuge.

a) Preparation of the annealing mixture

In a sterile PCR tube, 3.0 µg of total RNA (extracted in section 2.3.2), 1.0 µl of G1 and RNase-free H₂O were added to make a final volume of 10 µl. The contents were mixed well followed by a brief centrifugation to collect the mixture at the bottom of the tube. Samples were incubated at 70°C for 10 min, then centrifuged briefly and placed on ice.

b) Preparation of the cDNA synthesis master mix was shown in Table 5.

Table 5.

cDNA synthesis master mix	Per reaction
RNase-free H ₂ O	4 µl
5 X cDNA Synthesis Buffer (G3)	4 µl
RNase Inhibitor (RI)	1 µl
cDNA Synthesis Enzyme Mix (G2)	1 µl
Final volume	10 µl

The components were combined in the order listed above, and mixed well with pipetting up and down two to three times followed by brief centrifugation to collect the mixture at the bottom of the tube. The cDNA synthesis master mix was then placed on ice.

c) cDNA Synthesis reaction: To each tube containing 10 µl of annealing mixture,

10 μ l of cDNA synthesis master mix was added. The components were mixed well and then centrifuged briefly. Each reaction tube was incubated at 42°C for 50 min followed by 75°C for 5 min then cooled to 37°C.

2.3.6 cRNA synthesis, labeling and amplification

a) Preparation of the amplification master mix was shown in Table 6.

Table 6.

Amplification master mix	Per reaction
2.5 X RNA Polymerase Buffer (G24)	16 μ l
Biotin-UTP	2 μ l
RNA Polymerase Enzyme (G25)	2 μ l
Final Volume	20 μ l

The above components were well mixed with pipetting up and down two to three times followed by brief centrifugation.

b) cRNA Synthesis reaction: To each tube containing 20 μ l of cDNA synthesis reaction, 20 μ l of amplification master mix was added. The components were mixed gently and centrifuged briefly. Then the reaction tubes were incubated overnight at 37°C.

2.3.7 cRNA purification

a) Binding cRNA to the Spin Column: A spin column was set up in a collection tube for each sample. RNase-free H₂O (60 μ l) was added to each cRNA synthesis reaction tube for a final volume of 100 μ l. The entire reaction mixture was transferred to one 1.5-ml RNase-free tube. Lysis & binding buffer (G6) (350 μ l) was added to each of the reaction mixture. The contents were mixed well with gentle pipetting for two to three times. Then 350 μ l of room temperature ACS-Grade 100% ethanol was added and contents were mixed well with gently pipetting. Each sample was loaded to the center of its own spin column and centrifuged for around 30 sec at 8,000 x g. The column was removed from the tube and the flow-through was discarded.

b) Washing the spin column: To each spin column, 600 μ l of washing buffer (G17 with ethanol) was applied. The column was centrifuged for about 30 sec at 8,000 x g. The column was removed and the flow-through was discarded. The column was placed back into the collection tube. Then 200 μ l of washing buffer (G17 with ethanol) was applied to each spin column, and the column was centrifuged for 3 min at 11,000 x g.

c) Eluting the cRNA from the spin column: Each spin column was transferred to

a fresh elution tube. To the center of each spin column, 50 μ l of room temperature RNase-free 10 mM Tris buffer pH 8.0 (G26) was added. The spin column was incubated at room temperature for 2 min, followed by centrifugation for 1 min at 8000 x g. After the entire volume of buffer passed through the filter, the purified cRNA was stored on ice.

d) cRNA quality assessment: The quality of cRNA was assessed by UV spectrophotometry as described previously.

2.3.8 Oligo GEArray hybridization

a) Pre-hybridization: To pre-wet the array membrane, roughly 5 ml of deionized water was added to the hybridization tube. The cap was screwed on hand-tight and the tube was allowed to sit inverted for 5 min. The GEAhyb Hybridization solution was warmed to 60°C and the bottle was inverted several times to allow complete dissolution of the buffer components. The deionized water was discarded from the hybridization tube and 2 ml of pre-warmed GEAhyb hybridization solution was added. The tube was briefly vortexed and placed in the hybridization cylinder. After checking that the warm hybridization tubes and cylinders were sealed hand-tight, the tubes were pre-hybridized in the hybridization oven at 60°C for 2 hours with continuous but slow agitation at 5 to 10 rpm.

b) Hybridization: At least 2 μ g of biotin-labeled cRNA target was added to 0.75 ml of pre-warmed GEAhyb hybridization solution and mixed well. Then the pre-hybridization solution was discarded from the hybridization tube and the target hybridization mix containing the labeled cRNA target was added to the tube. Hybridization was continued overnight at 60°C with slow agitation at 5 to 10 rpm.

c) Preparation of wash solutions: Wash solution 1 (2 X SSC, 1% SDS) was prepared by mixing 10 ml of 20 X SSC with 5 ml of 20% SDS and 85 ml of dH₂O. Wash solution 2 (0.1 X SSC, 0.5% SDS) was prepared by mixing 0.5 ml of 20 X SSC with 2.5 ml of 20% SDS and 97 ml of dH₂O. Both wash solutions 1 and 2 were warmed to 60°C before use. The preparations of 20 X SSC and 20% SDS were as follows in Table 7:

Table 7.

20 X SSC (pH 7.0)	
Sodium Chloride (NaCl)	2.63 g
Sodium citrate dihydrate	1.323 g
Distilled water	10 ml
20% SDS	

Sodium dodecyl sulfate (SDS)	200 g
Distilled water	1 L

d) Washing: The target hybridization mix was transferred to a clean microcentrifuge tube and 5 ml of wash solution 1 was added to the hybridization tube. The tube was placed back into the hybridization oven and the membrane was washed at 60°C with faster agitation at 20 to 30 rpm for 15 min. The wash solution was discarded and 5 ml of wash solution 2 was added. The membrane was washed for 15 min at 60°C with 20 to 30 agitations. The wash solution was immediately discarded and the cap was placed back on the hybridization tube to prevent membrane from drying. The tube was allowed to cool to room temperature.

2.3.9 Chemiluminescent detection

a) Blocking the Oligo GEArray: GEAblocking solution Q (2 ml) was added to the hybridization tube and vortexed briefly. The tube was incubated for 40 min with continuous agitation at 20 to 30 rpm.

b) Preparation of solutions

- i. 1 X Buffer F: 5 X Buffer F was diluted five-fold with dH₂O.
- ii. Dilute AP-SA Buffer: AP-SA was diluted in 1:8,000 with 1 X Buffer F. For 8 Oligo GEArrays, 2 µl of AP-SA was added into 16 ml of 1 X Buffer F.

c) Binding of alkaline phosphate-conjugated streptavidin (AP): After GEAblocking solution Q was discarded from the tube, 2 ml of diluted AP-SA buffer was added and incubated for exactly 10 min with continuous but gentle agitation (5-10 rpm).

d) Washing: The membrane was washed four times with 4 ml 1 X Buffer F for 5 min with gentle agitation. The tube was well vortexed after each addition of fresh 1X Buffer F.

e) Rinsing: After the last wash was discarded, 3 ml of Buffer G was added and the tube was inverted three times. The buffer was discarded and the procedure was repeated twice.

f) Detection: After rinsing, 1.0 ml CDP-Star chemiluminescent substrate was applied to the hybridization tube. The tubes were rotated in room temperature hybridization oven for 3 to 5 min.

g) Image acquisition: After the CDP-Star incubation, the GEArray was ready for

image acquisition. To remove excess CDP-Star solution, one corner of the array membrane was held by forceps and the opposite membrane corner was in contact with a piece of clean, absorbent paper. The membrane was placed between two plastic transparencies and image was acquired on a Super RX film (Fuji).

h) Microarrays: After obtaining exposures, the damp membrane was returned back to its original plastic disposable hybridization tube. The cap was placed hand-tight and stored at -20°C.

2.3.10 Data analysis

Image, data and statistical analysis were performed with GEMatrix[®] Expression Analysis Suite Package. After logging into the site, <http://geasuite.superarray.com/>, project name was created and Array image was uploaded to each project. Images were optimized and adjusted to the crop frame in the Image Setting Workplace. Then workplace was switched to Grid/Readout where circles on the grid were aligned to the spots on the image. When the position and size of the spots were adjusted, readout was built and saved. Once readouts were available for all arrays, analysis was done to Arrays in two groups and gene names and their expressions were listed. A Scatter plot was obtained from all Arrays in one group and fold changes in gene expression between groups were calculated.

Materials & Methods -- *In vivo*

Andrographolide is believed to have pharmacological activity. In this part of the experiment, the effect of andrographolide on hepatocarcinoma in rats was investigated through ALT & AST assays, hematoxylin & eosin staining, immunohistochemical staining of GST-P, RNA expressions of *p53* & *mdm2* and western blot analysis of p53, mdm2, PCNA, Bax, Bcl-2 and p21.

EXAMPLE 3. Effects of andrographolide on hepatocarcinogenesis in rats

Animal Treatment, liver perfusion, AST/ALT Assays & Stainings

3.1 Materials & Solutions

Table 8. Materials & solutions as used

Chemicals & Reagents	Purchasers
ALT/SGPT (UV-Rate) & AST/SGOT (UV-Rate) Kits for detection of alanine and aspartate transferases respectively	Stanbio Laboratory (Texas, USA)
Diethylnitrosamine (DEN), hydrogen peroxide (30%), diaminobenzidine (DAB), formaldehyde 36%	Sigma Chemicals (St. Louis, USA)
Anti-GST-P polyclonal antibody (rabbit)	Medical & Biological Laboratories
Biotinylated goat anti-rabbit IgG and avidinbiotin-peroxidase complex (ABC Staining System)	Santa Cruz Biotechnology (Ca., USA)
Superfrost / plus microscope slide (25 x 75 x 1.0 mm), DMSO (Dimethylsulfoxide)	Fisher Scientific (Pittsburgh, USA)
Glass microscope slide (25.4 x 76.2 x 1.2 mm)	Sail Brand (Shanghai)
Ethanol 100%	BDH (England)
Carbon tetrachloride (CCl ₄)	Merck (Germany)
Tris, sodium dodecyl sulfate (SDS), citric acid (trisodium dehydrate)	USB (Cleveland, USA)
Andrographolide (AND) 98%	Sigma-Aldrich (USA)
Corn Oil	Vecorn (USA)

3.2 Preparation of Solutions

DEN solution (200 mg/ml): DEN solution for the initiation of rat HCC was prepared by diluting 2.52 ml of DEN to 12 ml with DMSO.

CCl₄ solution: It was prepared by mixing CCl₄ with corn oil in 1:1 ratio.

Andrographolide (10 mg/ml) solution: It was prepared by dissolving 500 mg of AND in 50 ml of corn oil.

Tris-sucrose buffer (pH 7.4): The buffer used for liver perfusion was prepared by dissolving 95.6 g of sucrose and 6.06 g of Tris in 900 ml of dH₂O. The pH was adjusted to be 7.4 with HCl and volume was made up to 1000 ml with dH₂O. The solution was then stored at 4°C.

Formaldehyde solution (10%): Formaldehyde buffer for the liver section fixation was prepared by mixing 10 ml of 36% formaldehyde with 26 ml of dH₂O to make 36 ml of formaldehyde solution.

1% hydrogen peroxide (H₂O₂): The solution was freshly prepared by diluting 3 ml of 30% hydrogen peroxide with dH₂O to make a final volume of 300 ml.

Citric acid buffer (pH 6.0): It was prepared by dissolving 0.63 g of citric acid with 300 ml of dH₂O. The pH was adjusted to 6.0 with sodium hydroxide (NaOH).

Diluted normal serum (NS): The solution was prepared by diluting 150 µl of NS from ABC staining kit in 10 ml of 1 X PBS.

Diluted anti-GST-P antibody (1:10): This primary antibody was diluted in 1:10 ratio by adding 0.5 ml of anti-GST-P antibody to 4.5 ml of diluted NS.

Diluted Biotinylated goat anti-rabbit IgG (1:200): It was prepared by diluting 25 μ l of antibody in 4.975 ml of diluted NS.

Diluted avidinbiotin-peroxidase complex (ABC): It was prepared by mixing 100 μ l of A and B from ABC staining kit in 4.8 ml of 1X PBS.

DAB solution: It was freshly prepared by dissolving 15 mg of DAB in 150 ml of PBS, with 8 μ l of 30% H₂O₂ added before use.

3.3 Procedures

3.3.1 Animal treatment

Male Sprague Dawley rats (80-90 g of body weight) were obtained from Laboratory Animal Services Center of The Chinese University of Hong Kong (Hong Kong SAR, The People's Republic of China). They were divided into three groups (Negative control, Positive control and Treatment) and housed in three different cages in animal room maintained with a 12 hr light-dark cycle (06:00-18:00) at constant temperature and humidity of $55 \pm 5\%$. They were given unlimited rodent diet (Supastok Autoclavable Rodent Diet, Ridley Agriproducts, Australia) and water *ad libitum*. Rats were observed during the acclimatizing week and were then used for different treatments according to the protocols described below.

Table 9. Treatment of rats for the promotion & progression stages of hepatocarcinogenesis

	i.p. injection of toxicant/ vehicle (once a week)	Oral treatment (once a day)
Negative Control	DMSO + Corn Oil	Corn Oil
Positive Control	DEN + CCl ₄	Corn Oil
AND-treated	DEN + CCl ₄	Andrographolide (10 mg/kg)

Table 9 showed the treatment of rats in two stages of hepatocarcinogenesis. Rats were divided into three groups: negative, positive and AND-treated. The negative control group received i.p. injection of the vehicle, DMSO, and oral treatment of corn oil. The positive control and AND-treated groups received i.p. injection of a tumor initiator, DEN, for the first two weeks and then a tumor promoter, CCl₄, for the rest of the weeks. Rats in the positive control group were given corn oil once daily whereas those in the AND-treated group were given 10 mg/kg of AND.

3.3.2 Promotion (Experiment 1):

This experiment was designed to study the effect of AND at the promotion stage of rat hepatocarcinogenesis. Fifteen Sprague Dawley rats were equally divided into negative control, positive control and AND-treated groups. The negative control group received weekly intraperitoneal injection of DMSO (1 ml/kg of body wt) for the first two weeks, followed by a 5-day period of fasting for the next two weeks. Corn oil (1 ml/kg of body wt) was then administered intraperitoneally for the rest of the weeks. On the other hand, both the positive control and AND-treated groups received DEN (200 mg/kg body wt) for the first two weeks and then two 5-day periods of fasting. Between two weeks' of fasting, two days of *ad libitum* feeding were applied to allow maximum fasting experience with minimal fatalities. After the fasting periods, CCl₄ was administered intraperitoneally for the rest of the weeks. From the 5th week onwards, both the negative and positive controls received oral treatment of corn oil once a day. AND (10 mg/kg of body weight) was administered orally to the treatment group once daily. At the end of the experiment, which lasted for 28 weeks, all rats were weighed and then killed by nitrogen gas. Blood samples were collected. The liver samples were perfused with cold Tris-sucrose buffer and then removed, washed and weighed. Liver slices of about 2-3 mm in thickness were cut out. They were fixed in 10% formaldehyde for 24 hrs and then 75% ethanol until sections were embedded in wax by standard procedures. The remaining part of the liver was stored at - 80°C for further analysis.

3.3.3 Progression (Experiment 2):

This experiment was designed to investigate the effect of AND at the progression stage of rat hepatocarcinogenesis. Fifteen Sprague Dawley rats were equally divided into negative control, positive control and AND-treated groups. The intraperitoneal treatments for all the groups were the same as those in experiment I. Oral treatment with corn oil (1 ml/kg of body weight) or AND (10 mg/kg of body weight) was not given to the groups until the 32nd week of the experiment. At the end of the 56th week, rats were weighed and killed by nitrogen gas. Blood samples were collected for further analysis. Liver samples were perfused and stored as described in experiment I.

Treatment schedule of the rat in the promotion stage of hepatocarcinogenesis is shown in Figure 29. Group I represents the negative control rats which did not receive any toxicants. Group II is the positive control rats which were exposed to DEN and CCl₄. Group III represents the AND-treated group which was not given treatment until the 5th

week of the experimental schedule.

The schedule of the rat treatments in the progression stage of hepatocarcinogenesis is shown in Figure 30. Group I represents the negative control rats which did not receive any toxicants. Group II is the positive control rats which were exposed to DEN and CCl₄. Group III represents the AND-treated group. Oral treatments for the three groups were not given until the 32nd week of the experimental schedule.

Measurement of serum ALT and AST

3.3.4 Extraction of blood serum

Blood samples collected from rats were transferred to Vacutainers and were allowed to sit on ice for 10 min. After that, samples were centrifugated for 15 min at 4,000 rpm. Serum was extracted and was put on ice for ALT and AST analysis.

3.3.5 Measurement of absorbance

Activities of ALT and AST in rat serum were measured using Stanbio GP- and GO- Transminase Kits. The ALT kit (Procedure No. 0930) contained 13 mmol/L of α-ketoglutarate and 0.4 mol/L of DL-alanine. The AST kit (Procedure No. 0920) contained 12 mmol/L of 2-oxoglutarate and 0.2 mol/L of L-aspartic acid. The reagent was reconstituted with 15 ml of dH₂O and 1 ml of reconstituted reagent was added to cuvet and pre-warmed to 37°C for 3 min. Serum (0.1 ml) was added to the cuvet and was mixed gently by pipetting up and down several times. The content was warmed at 37°C for exactly 1 min. After the incubation, cuvet was placed into a spectrophotometer and was blanked at time zero. The absorbance was measured at 340 nm and readings were recorded at 30-second interval for 3 min. The amounts of ALT and AST were calculated as follows:

$$U/L = \frac{\Delta A / \text{Min}}{\text{Absorptivity}} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

$$U/L = \Delta A / \text{Min} \times 1768$$

Histological Analysis: H&E and immunohistochemical stainings

3.3.6 Tissue processing

Liver slices that were fixed in 75% ethanol were embedded in wax by tissue processor according to standard procedures. The waxed tissue samples were trimmed until

the whole surface of sample was exposed. Samples were cut on a microtome at 5 μm thick and were put into a 40°C water bath for a few minutes. Then two to three sections of each sample were mounted on glass slides for hematoxylin and eosin (H&E) staining and on superfrost plus microscope slides for immunohistochemical staining, respectively.

Slides, each containing two to three liver sections, were placed in a slide holder and warmed to 48°C in oven for 10 minutes. Sections were then dewaxed and rehydrated with xylene (5 min x 3), 100% ethanol (5 min x 3), 95% ethanol (1 min), 80% ethanol (1 min), 70% ethanol (1 min) and water (5 min).

3.3.7 Hematoxylin and Eosin (H&E) Staining (Nucleus & Cytoplasm)

Glass slides containing liver samples were stained in hematoxylin for 3 min. They were rinsed with tap water and allowed stain to develop for 5 min. After that they were destained with acid alcohol for 3 sec, followed by rinsing with tap water. Sections were placed in Scott tap for 1 min and then rinsed briefly in tap water. Then, slides were stained in eosin for 4 to 5 min, followed by rinsing with tap water. Sections were dehydrated briefly in 70% and 80% ethanol, and then 95% ethanol for 1 min, 100% ethanol for 5 min for 3 times, and lastly xylene for 5 min for 3 times. Finally slides were mounted with coverslip and Permount.

3.3.8 Immunohistochemical staining of GST-P

Superfrost plus microscope slides containing two to three sections of liver samples were dewaxed and rehydrated as described previously. Endogenous peroxidase activities of liver samples were blocked in 1% H_2O_2 for 5 min at room temperature. Sections were washed in tap water for 5 min, followed by antigen retrieval in 300 ml of citric acid buffer. The case and the buffer were first pre-warmed in microwave for 5 min. After the slides were placed in the buffer, sections were further heated for another 5 min on a hot plate. Sections were washed in tap water for 5 min and excess water was wiped off gently around each section. About 150-200 μl of diluted NS was added to each section to block the unspecific antigens and sections were allowed to incubate for 1 hr at room temperature. After the excess NS was drained off from the slides, sections were covered with 150-200 μl of anti-GST-P polyclonal antibody in 1:10 ratio and incubated overnight at 4°C inside moist chamber. After binding with primary antibody, sections were washed in PBS three times for 5 min each. About 200 μl of biotinylated goat anti-rabbit IgG diluted in 1:200 was added to each section and incubated for 30 min at room temperature.

The slides were washed with PBS for three changes, each for 5 min, and then covered with 200 μ l of freshly prepared ABC for 30 min at room temperature. After washing with PBS with three changes for 5 min each, sections were immersed in DAB solution for approximately 3 min, and positive areas could be visualized under microscope. Sections were washed with tap water for 5 min, followed by counterstaining in hematoxylin for 2 min. Then the slides were washed with tap water, and were briefly destained with acid alcohol for 2 sec. Slides were rinsed with tap water, then immersed in Scott tap for about 30 sec, and again rinsed with tap water. The slides were briefly dehydrated in 70% and 80% ethanol, and then 95% ethanol for 1 min, 100% ethanol for 5 min for 3 times, and lastly xylene for 5 min for 3 times. Finally, slides were mounted with coverslip and Permount.

3.3.9 Examination of liver sections

Liver sections were examined using an Axiophot-2 Universal microscope (Zeiss) coupled with Spot 32 image analysis system. Sections were viewed under 5x, 10x, 20x and 40x magnifications and were analyzed using Image J program.

EXAMPLE 4. Effects of Andrographolide on the expressions of Mdm2, p53, PCNA, Bax, Bcl-2 and p21

mRNA & protein extraction from liver, RT-PCR, SDS-PAGE & Western blots

4.1 Materials and Solutions

Table 10. Materials & solutions as used

Agarose, TEMED, HEPES, diethyl pyrocarbonate (DEPC), ammonium persulfate (APS), acrylamide/bis, MOPS, glycerol, bromophenol blue, sodium dodecyl sulfate (SDS), 5X M-MLV reaction buffer, M-MLV reverse transcriptase	USB
Chloroform, EDTA, isopropanol, formaldehyde, tris, magnesium chloride ($MgCl_2$), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), triethanolamine-HCl, beta-mercaptoethanol, deionized formamide, triton x-100, ethidium bromide (EB)	Sigma
Oligo dT primer, 10 mM dNTP mix, Taq polymerase, β -actin primer mix	Invitrogen
Trizol reagent, PCR buffer	Gibco BRL
Target gene primers	Tech Dragon Ltd
Complete 20 tablets (protease inhibitors)	Roche
Millipore Immobilon 26.5x375 cm Roll	Bio-Gene

	tech. Ltd
3MM Filter paper	Whatman
Tween-20	Pharmacia Biotech
Primary antibodies: anti- β -actin, anti-mdm2 (SMP14), anti-p53 (Pab246 & 421), anti-Bcl-2 (c-2), anti-Bax (5B7), anti-PCNA (pc10) and anti-p21 mouse monoclonal Secondary antibody: goat anti-mouse HRP labeled antibody	Santa-Cruz Biotech Inc
Methanol (MeOH), ethanol (EtOH)	BDH
Protein A sepharose CL-4B, ECL-Western Blotting detection reagent	Amersham
Fuji Super RX Film	Fuji Photo Products Co. Ltd

4.2 Preparation of solutions

25 mM MgCl₂: It was prepared by dissolving 0.119 g of MgCl₂ in 50 ml of d H₂O.

Solution A for nuclear protein extraction: The solution contained buffer A and 1 mM of PMSF.

Buffer A: It was prepared by dissolving 438.3 mg of NaCl, 119.15 mg of HEPES, 18.61 mg of EDTA Na₂ 2H₂O, and 0.3 g of Triton X-100 in 40 ml of water. The pH was adjusted to 7.9 with 1 M NaOH. The solution was made up to 50 ml of water, and then stored at 4°C.

PMSF solution (200mM): It was prepared by dissolving 348.4 mg of PMSF in 10 ml of isopropanol, and it was stored at -20°C.

Solution B for nuclear protein extraction: The solution contained 50 ml of Buffer B, 250 μ l of PMSF, 50 μ l of DTT and one complete tablet.

Buffer B: It was prepared by dissolving 238.3 mg of HEPES, 1227.2 mg of NaCl, 5.712 mg of MgCl₂, 3.722 mg of EDTA Na₂ 2H₂O, in 35 ml of water. The pH was adjusted to 7.9 with NaOH, and then 12.5 ml of glycerol was added. The solution was made up to a final volume of 50 ml with dH₂O and stored at 4°C.

1M DTT stock solution: DTT (1.5424 g) was dissolved in 10 ml of water and stored at - 20°C.

Solution C for whole cell lysate: It contained 50 ml of solution C, 250 μ l PMSF, 62.5 μ l DTT and one complete tablet.

Buffer C: It was prepared by dissolving 238.3 g of HEPES, 1227.2 mg of NaCl, 5.712 mg of MgCl₂, 3.722 mg of EDTA Na₂ 2H₂O in 35 ml of water. The pH was adjusted to 7.9 with NaOH. Then 0.3 g of Triton X-100 and 12.5 ml of glycerol were added.

The solution was made up to 50 ml of water and stored at 4°C.

Immunoprecipitation (IP) buffer: It contained 20 mM triethanolamine-HCl, 0.7 M NaCl, 0.5% (v/v) Nonidet P-40, 4.6 mM sodium deoxycholate, 1 mM PMSF, and one complete protease inhibitor tablet. The buffer was adjusted to 7.8 with HCl.

Borate buffer: It contained 0.1 M boric acid, 0.1% (v/v) Nonidet P-40, 3.1 mM sodium azide. The pH was adjusted to 8.0 with NaOH.

2 X SDS sample loading buffer: The buffer contained 2.5 ml of Tris HCl (1M, pH 6.8), 10 ml of 10% SDS, 0.00625 g of bromophenol blue, 5 ml of glycerol and 2.5 ml of beta- mercaptoethanol (14.4 M) in dH₂O to a final volume of 25 ml.

1 M Tris-HCl (pH 8.8): It contained 12.114 g of Tris in 80 ml of dH₂O. The pH was adjusted to 8.8 with HCl. The solution was made up to 100 ml with dH₂O.

1.5 M Tris-HCl (6.8): It was prepared by dissolving 18.171 g of Tris in 60 ml of dH₂O, and pH was adjusted to 6.8 with HCl. The solution was made up to 100 ml with dH₂O.

10% SDS: It contained 10 g of SDS in 100 ml of dH₂O.

30% Acrylamide/Bis: It was prepared by mixing 29 g of acrylamide and 1 g of bis- acrylamide in 100 ml of dH₂O.

10% ammonium persulfate: It contained 0.1 g of ammonium persulfate in 1 ml of dH₂O.

1 X running buffer: The buffer contained 3.02 g of Tris-base, 18.8 g of glycine and 10 ml of 10% SDS in 990 ml of dH₂O.

Transfer buffer: It contained 200 ml of MeOH, 2.93 g of glycine, 5.82 g of Tris and 3.75 ml of 10% SDS in 796.25 ml of dH₂O.

Tris-buffered saline/Tween (TBST) buffer: It was made up of 10 mmol of Tris-HCl (pH 7.5), 150 mmol NaCl and 0.1% Tween-20.

Blocking solution (5%): The blocking solution was prepared by dissolving 0.2 g of non-fat milk in 4 ml 1X TBST.

4.3 Procedures

Semi-Quantitative RT-PCR Analysis of mRNA expression

4.3.1 Total mRNA extraction from liver

The liver tissue (25 to 50 mg) was homogenized with 0.5 ml of Trizol reagent. The extraction of mRNA was performed as described in sections 2.3.2 to 2.3.4.

4.3.2 Reverse transcription of mRNA to cDNA

It was done by mixing 3 µg of RNA, 0.4 µl of oligo dT primer and autoclaved dH₂O to a final volume of 14 µl. Then the solution was incubated at 70°C for 10 min. After that, 1 µl of dNTP, 4 µl of M-MLV reaction buffer and 1 µl of M-MLV reverse transcriptase were added to the solution to make a final volume of 20 µl. The reverse transcription was carried out at 42°C for 50 min in a Perkin-Elmer GeneAmp[®] PCR system 9700. Samples were denatured for 15 min at 70°C and then cooled on ice.

4.3.3 Protocol for polymerase chain reaction (PCR)

The PCR reactions were performed in a final volume of 20 µl in a GeneAmp[®] PCR system 9700. Each PCR reaction contained 1 µl of cDNA (synthesized from 4.3.2), 2 µl of PCR buffer, 1.2 µl of MgCl₂, 1 µl of primer mix, 0.2 µl of Taq polymerase, 0.4 µl dNTP and 14.2 µl autoclaved dH₂O to a final volume of 20 µl. The PCR mixture was incubated at 94°C for 5 min followed by 30 to 35 cycles of amplification. The number of cycles of each gene was determined from a series of PCR reactions with different cycle numbers. For β-actin and mdm2, each of the 30 cycles consisted of 45 sec of denaturation at 94°C, 45 sec of annealing at 55°C and 30 sec of extension at 72°C. For p53, 35 cycles were performed and each cycle consisted of 45 sec of denaturation at 94°C, 45 sec of annealing at 58°C and 90 sec of extension at 72°C. When all cycles had been completed, a final extension step of 72°C for 10 min was performed.

Table 11. Primer Sequences for RT-PCR

Primer Name	Orientation	5' Primer sequence 3'
p53-F	Forward	GTGG ATCC TGAA GACT GGAT AACT GTC
p53-R	Reverse	AGTC GACA GGAT GCAG AGGC TG
Mdm2-F	Forward	GTCT CTGG ACTC GGAA GATT AC
Mdm2-R	Reverse	AAAC AATG CTGC TGGA AGTC G
β-actin-F	Forward	ACA CCT CAA ACC ACT CCC AG
β-actin-R	Reverse	AAC TCC TAA GGG GAG GAT GG

4.3.4 DNA Gel Electrophoresis

The PCR products were ready for gel electrophoresis which was performed as described in section 1.3.5.

Analysis of Protein expressions

4.3.5 Nuclear protein extraction

The nuclear protein extraction was performed by homogenizing 300 mg of liver tissue in 0.9 ml of solution A (buffer A and 125 μ l of 200 mM PMSF stock solution) on ice. The mixture was subsequently transferred to a microcentrifuge tube and centrifuged for 30 sec at 2,000 rpm at 4°C to get rid of any unbroken tissue. After that, the supernatant was incubated for 5 min on ice and centrifuged for 5 min at 5,000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in solution B and incubated on ice for 20 min for high-salt extraction. The lysed nuclei were transferred to a new microcentrifuge tube and centrifuged at 12,000 rpm for 30 sec to pellet the cellular debris. The supernatant was transferred to another microcentrifuge tube and was stored at -20°C for the detection of p53 and mdm2.

4.3.6 Cytosolic protein extraction

A 300 mg of liver samples were homogenized on ice in 0.9 ml of solution C and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was stored at -20°C for the detection of expression of Bax, Bcl-2, p21 and PCNA.

4.3.7 Determination of protein concentration

The protein concentration was determined by spectrophotometry at 280 nm. After correcting the protein concentration with the dilution factor, 2 X SDS-PAGE sample loading buffer was added to adjust the protein concentration to 100 mg/ml. Samples were boiled for 5 minutes, mixed by vortexing and spinned down.

4.3.8 Immunoprecipitation of p53 from liver nuclear protein

For immunoprecipitation, the protein A-sepharose beads were first activated by suspending 1 g of dry beads in 4 ml of borate buffer and mixing for 1 hr. The borate buffer was centrifuged for 15 sec at 15,000 g and it was removed. The beads were resuspended in borate buffer to obtain 50% (v/v) suspension, and stored at 4 °C.

The wild type anti-p53 (Pab 246) monoclonal antibody (2 μ g), dH₂O (100 μ l), nuclear protein (200 μ g), and 2 X IP buffer (100 μ l) were added to a microcentrifuge tube and incubated for 1 hr with agitation at 4°C. The goat anti-mouse IgG antibody (5 μ g) was added and the solution was further incubated for another 30 min. Then, 50% protein A-sepharose beads (50 μ l) were added and incubated for 30 min at 4°C with agitation. The protein A-sepharose beads were recovered by centrifugation (15 sec at 15,000 g) and

washed with IP buffer by centrifugation (15 sec at 15, 000 g) and resuspended in 400 μ l of IP buffer. The pellet was resuspended in 30 μ l of 2 X SDS-PAGE sample loading buffer and was loaded onto an SDS-PAGE gel for electrophoresis separation after boiling.

4.3.9 Protein gel electrophoresis by SDS-PAGE

The electrophoresis system, Mini-PROTEAN[®] II cell from Bio-Rad, was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A gel casting mould was assembled and tested with dH₂O for leakage. The resolving gel solution (10%) was set and poured into the gel casting form. The top of the gel was layered with dH₂O and the gel was allowed to polymerize for about 30 min. Water was discarded and stacking gel solution (4%) was poured on top of the resolving gel. A 10-tooth comb was inserted and the gel was allowed to polymerize for another 30 min. Samples mixed with 2 X sample loading dye were boiled at 95°C for 5 min. After samples were loaded into the wells, the gel was run at constant voltage at 150 V for 1 hr in 1 X running buffer.

Table12. Components of resolving and stacking gels in SDS-PAGE

	Resolving gel 10% (1 gel)	Stacking gel 3% (1 gel)
dH ₂ O	3.6 ml	2.55 ml
Tris	2.25 ml (1 M, pH 8.8)	0.48 ml (1.5 M, pH 6.8)
30% Acrylamide/Bis	2.96 ml	0.68 ml
10% SDS	75 μ l	37.5 μ l
10% (w/v) AP	75 μ l	37.5 μ l
TEMED	3.5 μ l	3.75 μ l

4.3.10 Western blotting

After SDS-PAGE was completed, the gel was removed and immersed into transfer buffer. Whatman 3 MM paper (6 pieces per gel) and PVDF membrane were cut into the same dimension as the resolving gel. Before transfer, the PVDF membrane was rinsed briefly with 100% MeOH for around 1 min and then immersed into transfer buffer. The 6 pieces of Whatman papers were also soaked into transfer buffer before use. Semi-dry Trans-Blot electroblotter (Bio-Rad) was used for the protein transfer. A gel sandwich was assembled with the PVDF membrane and the resolving gel layered between two stacks of 3 Whatman papers. Proteins were transferred to the membrane at constant voltage at 10 V for 1.5 hr.

When the transfer was completed, the sandwich was disassembled and the

membrane was rinsed briefly with TBST buffer. The membrane was immersed into blocking solution with primary antibody for 16 hr at 4°C with continuous agitation. The primary antibody used was mouse monoclonal anti-p53 (1:1,000), anti-mdm2 (1:500), anti-p21 (1:500), anti-Bcl-2 (1:500), anti-Bax (1:500) and anti- β -actin (1:5,000). The unbound primary antibody was washed away with TBST for 15 min. After three washings, the membrane was immersed into secondary antibody for 1 hr at room temperature with slow agitation. The secondary antibody used was goat anti-mouse HRP labeled antibody and was diluted in 1:10,000 with blocking solution. The excess antibody was washed away with TBST three times (15 min each). ECL-Western Blotting detection reagents (0.5 ml of each reagent) were mixed together and were applied to the membrane for 3 to 5 min. After removing excess reagent, protein bands on the membrane were visualized and recorded on a Super RX film (Fuji). Intensities of the bands were analyzed using Image J program.

Results – *In vitro*EXAMPLE 1: Effects of andrographolide on Cell Viability and Cell Cycle

The effect of andrographolide on cell cycle was investigated. Neutral red assays were performed to obtain the IC_{50} of cells treated with different concentrations of AND for 24, 48 and 72 hrs. Treatment of HepG2, the human liver cancer cells, with different concentrations of AND (0 to 100 μ M) showed that AND could inhibit cell viability by 50% with concentrations 17.5, 16 and 17 μ M with respect to 24, 48 and 72 hrs of incubation (Fig. 1). The effect of AND on normal liver cells was also investigated. WRL 68, the human normal liver cell line, was found to be inhibited by 50% with 57, 30 and 35 μ M of AND incubated for 24, 48 and 72 hrs respectively (Fig. 2). The IC_{50} for Clone 9, the rat normal liver cells, was 22 at 24 hrs, 18 at 48 hrs and 18 μ M at 72 hrs of incubation (Fig. 3). The IC_{50} determined for HepG2 cells for all three time periods were significantly lower than those determined for WRL 68 cells ($P < 0.05$).

Flow cytometry was performed to study the cell cycle of HepG2 upon treatment of AND. The DNA profiles of HepG2 cells treated with 1% DMSO as the control and 20 and 25 μ M AND for 24 hrs were shown in Fig. 4. The number of cells counted was 10,000 and the population of cells suspended in each phase of the cell cycle was shown in percentage (Fig. 5). Upon treatment of AND, more cells were suspended in sub G_1 phase than those treated with DMSO only. There were no significant changes in the population of cells suspended in G_0/G_1 , S and G_2/M phases between AND-treated cells and control cells ($P > 0.05$). The DNA profiles obtained from PI staining of HepG2 cells treated with 1% DMSO and 12.5 and 25 μ M of AND for 48 hrs were shown in Fig. 6. Treatment of AND significantly induced the accumulation of cells in the sub G_1 and G_0/G_1 phases of the cell cycle when compared with the control cells (Fig. 7). The populations of cells suspended in S and G_2/M phases were also significantly decreased when compared with those in the control group ($P < 0.05$). The results suggest that AND-treated cells were unable to progress from the G_0/G_1 phase to the S phase. They arrested cell cycle progression in the G_0/G_1 phase, causing cell death through apoptosis.

A hallmark feature of apoptosis was the observation that nuclear DNA extracted from apoptotic cells was often degraded (86). Therefore, to further confirm the mechanism through which AND caused cell death, DNA was extracted from HepG2 cells treated with 12.5, 20, 25 and 50 μ M AND for 72 hrs. DNA was analyzed

using agarose gel electrophoresis and fragmentation at around 200 bp was detected in cells treated with 50 μ M of AND (Fig. 8). Therefore, it demonstrated that AND caused cell death through apoptosis.

EXAMPLE 2: Effects of Andrographolide on gene expressions

In an attempt to evaluate the toxicological aspect of AND, cDNA microarray analysis associated with human toxicology and drug metabolism was performed. This microarray generated the expression profiles of 263 genes related to the metabolic processes of cell stress, cell toxicity, drug resistance, and drug metabolism.

A scatter plot was generated from the arrays of the control and AND-treated groups (Fig. 9). The up-regulated genes included those whose expression level is critical in drug metabolism such as NAD(P)H dehydrogenase quinone 1 (NQO1), superoxide dismutase 1 (SOD1), thymidylate synthetase (TYMS) and xanthine dehydrogenase (XDH). Other up-regulated genes also included those that are involved in cell cycle regulation such as RB1.

The down-regulated genes included a number of apoptotic and cell cycle regulators such as BCL2, BCL2L2, and CHEK2. Many genes whose expression level is important to drug metabolism such as CYP 2D6 and CYP 2A6 were also down-regulated. Some genes related to cell growth, proliferation and differentiation were under-expressed. They included IL1B and NFkB2 (Fig. 10).

Results – In vivo

EXAMPLE 3: Effects of Andrographolide on hepatocarcinogenesis in rats

In this part of the experiment, the promotion and progression stages of hepatocarcinogenesis were investigated. The combined effects of DEN and CCl₄ was observed after 5 months of treatment. Gross examination of the liver showed that the liver had expanded from the right to the left side of the rat's abdomen (Fig. 11A). A closer look of the liver showed that there had been a change in morphology and nodules were observed across the liver surface (Fig. 11B). In the promotion experiment, the livers of the negative control group generally had a smooth surface and neither nodules nor lipids could be observed on the surface. On the other hand, the positive control livers had a thickened border and the surface was rough with lipid droplets distributing throughout the whole surface. The AND-treated livers had a smooth surface and generally resembled the negative control livers (Fig. 12). In the

progression experiment, no abnormal appearance of the rat liver was found in negative control group. However, it was observed that rat livers were hardened and nodules or lumps were present in positive control group. There were also some nodules present on the surface of the AND-treated livers, but the structure of the livers generally resembled that of the normal livers (Fig.13).

The relative liver weights of rats from the negative, positive and AND-treated groups in both experiments were compared (Fig. 14). There was a significant increase in the relative liver weights in positive control groups as compared with those of rats in negative control groups. A significant reduction in the relative liver weights was found in the AND-treated groups in both experiments. No significant difference was observed in AND-treated groups when compared with negative control groups in both experiments (Fig. 14).

Serum AST and ALT activities were measured to assess liver damage. The percentage changes of serum AST and ALT levels relative to negative control were summarized in Fig. 15. In both experiments, values for serum AST and ALT levels in rats of positive control group were elevated as compared with those in negative control group. There was a significant reduction in AST and ALT levels in rats that had treated with AND (Fig. 15A for promotion experiment, $p < 0.01$; Fig. 15B for progression experiment, $p < 0.05$). These data suggest that AND is effective in decreasing serum AST and ALT levels.

Histological examination of livers from rats in the promotion stage of hepatocarcinogenesis was carried out through Hematoxylin & Eosin (H & E) staining. The nuclei of hepatocytes were stained blue due to the color of hematoxylin and the cytoplasm was stained red due to eosin. The liver section of a negative control rat had a typical histological structure with a characteristic pattern of hexagonal lobules. The central vein was clearly observed and hepatocytes surrounding the central vein were arranged in columns, radiating from it (Fig. 16A, B). It was shown that after treatment of carcinogens, normal hepatocyte alignment was disrupted and cytoplasmic vacuolization within hepatocytes could be observed (Fig. 16C). The conditions were improved in those livers obtained from AND-treated rats. Liver structure was restored and hepatocyte arrangement was not as distorted as that of the positive control sections. Vacuolization was reduced and distinct cytoplasm was visible (Fig. 16D). In the progression experiment, the structural appearance of the negative control section was similar to that in the promotion group (Fig. 17A). The liver section of the rat treated

with AND also showed normal alignment of hepatocytes around central vein (Fig. 17B). The positive control sections, on the other hand, showed that hepatocytes were arranged randomly across the whole section and distinct cytoplasm could not be observed (Fig. 17C, 17D).

Immunohistochemical staining of GST-P foci was performed to detect the presence of neoplastic cells. Negative control rats in the promotion stages of hepatocarcinogenesis did not develop any GST-P positive liver foci (Fig. 18A, B). In carcinogen treated groups, GST-P foci were detected (Fig. 18C, D). The expression of GST-P was found in clusters or groups and they could be found across the whole liver section. In the AND-treated liver section, the expression of GST-P was present but the affected area was reduced (Fig. 18E, F). In the progression group, GST-P foci could not be detected in the normal rat liver sections (Fig. 19A). The rats in the positive control groups showed clusters of GST-P foci (Fig. 19B). A closer look at these sections showed cytoplasmic vacuolization and abnormal alignment of hepatocytes (Fig. 19C). In some areas of the positive control section where expression of GST-P was not detected, hepatocytes were aligned randomly and no distinct cytoplasm was present (Fig. 19D). The AND-treated liver sections were similar to the negative control sections with distinct central vein and normal hepatocyte appearance (Fig. 19E, F).

EXAMPLE 4. Effects of Andrographolide on the expressions of Mdm2, p53, PCNA, Bax, Bcl-2 and p21

To study the effect of AND on the inhibition of hepatocarcinogenesis in both the promotion and progression stages, Western blot of PCNA, a marker of cell proliferation was done (Fig. 20). The level of PCNA expression in DEN-CCl₄ treated rat was found to be significantly higher than that in the negative control and AND-treated groups in the progression experiment. However, there was no significant difference in the levels of PCNA expression in the AND-treated and positive control groups in the promotion experiment.

To determine the effect of AND on cells associated with apoptosis, the protein expressions of Bax and Bcl-2 were investigated by Western blot analysis (Figures 21-22). In both experiments, the levels of Bax protein expressed in the liver of rats which received carcinogens were significantly lower than those rats which received vehicle or AND treatment. The levels of Bcl-2 in the liver of

carcinogen-treated rats in the positive control groups were higher than that in the control or AND-treated groups. These data suggest that AND had triggered a reduction of Bax expression but an increase in Bcl-2 expression.

The effects of AND on cell cycle regulation were investigated through the Western blot analysis of p21 (Waf1/Cip1/Sdi1), which functions at major transition points in the cell cycle. In both the promotion and progression experiments, the levels of p21 (Waf1/Cip1/Sdi1) in carcinogen-treated groups were lower than those in the negative control and AND-treated groups. However, no significant difference could be observed among the three groups (Fig. 23).

The total p53 protein (wild type and mutant) expressions in rat liver nuclei were measured (Fig. 24). When compared with the negative control groups, higher levels of total p53 protein expression were found in rats treated with DEN-CCl₄ alone in the positive control groups and the AND-treated groups in both the promotion and progression experiments. However, no significant difference was observed among these groups. The wild type p53 functions in cell cycle arrest and in triggering the apoptotic event. The changes in the wild type p53 protein expression were determined through immunoprecipitation and western blot analysis (Fig. 25). In the promotion experiment, the level of wild type p53 protein expression was lower than that in the AND-treated and control rats. However, no significant difference was observed in these groups. In the progression experiment, nuclear wild type p53 was significantly reduced in the positive control group as compared to those in the negative or AND-treated groups. The results showed that administration of AND significantly increased the expression of wild type p53 compared with the positive control.

The protein expression of p53 was regulated by Mdm2. In both experiments, there was a significant overexpression of Mdm2 in DEN-CCl₄ treatment in positive control groups as compared with those that received the vehicle treatment. A significant decrease in the levels of Mdm2 was observed in AND-treated groups as compared with the positive control groups (Fig. 26). The findings suggest that treatment with AND significantly reduced the protein expression of Mdm2.

The effects of AND on the inhibition of hepatocarcinogenesis were further investigated at the transcriptional level of total *p53* and *Mdm2*. The transcripts encoding the mRNA of *p53* and *Mdm2* were normalized with the corresponding β -actin mRNA transcripts and changes in the transcriptional level were expressed in ratio as shown in Fig. 26 and Fig. 27. In both experiments, it was observed that the

mRNA expression of *p53* was low in the vehicle control groups. The treatment of DEN-CCl₄ significantly increased the mRNA transcripts of *p53*. Upon treatment of AND, there was a significant reduction in the total *p53* transcription expression which was brought about by the carcinogens. The mRNA expression of *Mdm2* was also low in the vehicle-treated rats as shown in Fig. 28. There was a considerable over-expression of *Mdm2* mRNA in carcinogen-treated positive control groups in both experiments. However, the expression of *Mdm2* mRNA was significantly reduced in AND-treated groups when compared with that in the positive control groups.

Discussion

Andrographolide (AND), the active component extracted from *Andrographis paniculata* (AP), was reported to possess pharmacological properties. These include protozoacidal, anti-mutagenic and anti-carcinogenic activities (82-85). The beneficial effects of AND on liver, however, are not fully understood. Therefore, the present study was aimed at investigating the biological activities of AND on the liver through *in vitro* and *in vivo* studies.

AND causes inhibition of cell growth

Cell cycle is an ordered set of events in a eukaryotic cell from one cell division to the next. It is initiated in the presence of mitogenic stimulus and is normally regulated by cyclins and cyclin-dependent kinases (87). However, this regulation of the cell cycle is lost in cancer cells and they continue to divide in the presence or absence of a mitogenic stimulus (88). Many anti-cancer agents are known to possess the ability to block the cell cycle at different stages. This includes doxorubicin, an anti-cancer agent that blocks cell cycle at G₂ phase (89).

In the present study, AND displayed a significantly inhibitory effect on the proliferation of human liver cancer cell line, HepG2 (Fig. 1). On the other hand, the normal liver cells, WRL 68 and Clone 9, were less sensitive to AND, although significant cytotoxicity was also detectable at a concentration of 18 μ M (Fig. 2 & 3). The results imply that AND could have therapeutic potential in treatment of cancer when lower doses are administered to rats.

In addition, FACS analysis demonstrated that the HepG2 cells treated with AND (25 μ M) for 48 hrs exhibited a dramatic accumulation (67.1%) of cells in G₀/G₁ phase of the cell cycle (Fig. 7). The increase in the population of cells suspended in

G₀/G₁ phase is regulated by many cell cycle proteins. The most critical determinant of the G₁ arrest is p21 (Waf1/Cip1/Sdi1) which interacts with cyclins D and E during the early G₁ phase of the cell cycle, inhibiting the activity of cyclin D/ CDK complex (42). The inactive cyclin D/CDK complex leads to the inactivation of kinases which subsequently activates the tumor suppressor, retinoblastoma (Rb), and that inhibits cell cycle progression through the G₁ to S phase. Besides the important role of p21, the expression of level cyclin D1 has also been shown to be rate-limiting in cellular proliferation (90). Consistent with its role in cell cycle progression, increased expression of cyclin D1 has been detected in breast cancer cells (91) and colorectal carcinogenesis (92). The tumor suppressor, Rb, functions as an active repressor of the transcription of E2F-responsive genes that are required for the initiation of DNA synthesis (93). Mutations of its DNA sequence or defect in gene transcription can result in Rb inactivation which occurs in all retinoblastomas (94). Therefore, p21, cyclin D1 and Rb are important elements in governing cell cycle progression at G₁-S-phase transition. In the present study, although no significant difference was observed, a higher expression of p21 was detected *in vivo* by Western blots in livers from negative control and AND-treated rats than that from the positive control rats. Microarray analysis further revealed that expression of *CCND1*, whose protein product is cyclin D1, was down-regulated whereas *RB1*, whose protein product is Rb, was up-regulated upon treatment of AND. Another cell cycle control gene, *CHEK-2*, whose expression level is critical to cell cycle checkpoint regulation and putative tumor suppression, was also found to be down-regulated. The findings show that AND can cause an arrest in the cell cycle at G₁-S phase transition in liver cells.

AND causes apoptotic cell death

Apoptosis is a genetically encoded form of cell suicide central to the development and homeostasis of multicellular organisms. Cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including nuclear disintegration (95). The cleavage of DNA into oligonucleosomal-length fragments is a late event in apoptosis and it was detected *in vitro* in the cell cycle analysis. The treatment of HepG2 cells with AND (25 μ M) resulted in a significant increase in the percentage of cells (13.1%) suspended in the Sub G1 phase of the cell cycle (Fig. 7). DNA fragmentation in HepG2 cells was further confirmed with 50 μ M of AND incubated for 72 hrs (Fig. 8).

Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic pathway and the extrinsic pathway converge at the mitochondrial level and trigger mitochondrial membrane permeabilization (96). After apoptotic-stimulated mitochondrial membrane permeabilization, cytochrome *c* and other proapoptotic proteins release into the cytosol. Released cytochrome *c* subsequently triggers the activation of caspases, substrate cleavage, and cell death (97). Bcl-2, and Bax are the two members of the Bcl-2 family that have been implicated as major regulators in the control of mitochondrial cytochrome *c* release (97). Bcl-2 is an anti-apoptotic factor that binds to the outer membrane of mitochondria and blocks cytochrome *c* efflux. Conversely, upon apoptosis induction, Bax translocates from the cytosol to the mitochondria where it enhances cytochrome *c* release through the outer membrane of mitochondria. Many anticancer agents or apoptotic stimuli can trigger cytochrome *c* release through either down-regulation of Bcl-2 and/or up-regulation of Bax such as Doxorubicin (98). In the present study, Bcl-2 protein expressions were significantly lower in both the negative and AND-treated rats than that in the positive control rats (Fig. 22). The protein levels of Bax were also significantly increased in the negative control and AND-treated groups than that in the positive control group (Fig. 21). Moreover, the microarray analysis revealed that *Bcl-2* and *Bcl-w*, an anti-apoptotic gene, were down-regulated in HepG2 cells treated with 16 μ M of AND for 48 hrs (Fig. 10). The data indicate that the anti-HCC effect of AND is associated with Bcl-2, Bcl-w and Bax proteins that trigger apoptosis.

AND increases the stability of wild-type p53

The tumor suppressor gene p53 is regarded as a key regulator in maintaining a balance between cell growth and cell death and it plays an important role in tumor growth inhibition and induction of apoptosis (99). The wild-type p53 mediates the expressions of Bax, Bcl-2 and p21 proteins. The results from *in vivo* study showed that wild-type p53 expression was decreased in the liver of DEN-CCl₄ treated rats, while total p53 (wild-type and mutant) expression was increased. It implies that the expression of mutant p53 was increased in the DEN-CCl₄ treated rats. The major regulator of p53 turnover, Mdm2, was found to be over-expressed at the transcriptional level in the livers of these rats (Fig. 26). This is due to the fact that Mdm2 expression is also induced by p53 through a binding site of the *Mdm2* gene. Since mutant p53 cannot be degraded by Mdm2, the over-expression of Mdm2 leads to an increase of wild-type p53 degradation.

The present data demonstrate a significant increase in the expression of p53 mRNA in the positive control rats, resulting in an increase in the total p53 protein level in both the promotion and progression stages of carcinogenesis. On the other hand, the p53 mRNA expression was found to decrease in the AND-treated group, and yet the total p53 protein was increased. Since wild-type p53 protein expression was significantly increased in the AND-treated rats and there was a significant decrease in the expression of Mdm2 simultaneously, the results suggest that AND down-regulates the expression of Mdm2, leading to an increased level of wild-type p53. It is confirmed with the microarray analysis, which showed a 3.4-fold changes in the down-regulation of *Mdm2* expression in HepG2 cells treated with AND (16 μ M) for 48 hrs (Fig. 10C). The under-regulated *Mdm2* gene expression reduces the protein level of Mdm2 to degrade wild-type p53, suggesting an increase in the stability of p53. Subsequently, the wild-type p53 can regulate the expressions of Bax, Bcl-2 and p21, resulting in cell cycle arrest and apoptosis in tumor cells.

AND reduces liver damage caused by DEN and CCl₄

Sprague Dawley rats were treated according to established protocols in order to investigate the effects of AND on the promotion and progression stages of hepatocarcinogenesis. The initial stage of HCC can be developed in rat liver by the administration of diethylnitrosamine (DEN), an indirect initiator that produces DNA adducts. Within a few weeks, populations of initiated cells called the enzyme-altered foci can be detected (100). With the administration of a promoter like CCl₄, the growth of the initiated preneoplastic cells is enhanced. Furthermore, the two periods of fasting in the treatment schedule were believed to reduce the latency period for the incidence of early lesion during chemical carcinogenesis in rat liver (101).

In generating a chemical carcinogenesis model in rat liver, liver cell proliferation plays a very important role in the whole process (102). It specifically exerts a critical effect in the promotion of carcinogen-initiated cells (103). Proliferating cell nuclear antigen (PCNA) is a co-factor for DNA polymerase δ and is synthesized in early G₁ and S phases of the cell cycle. It serves as a significant marker for proliferating cells and hence clinical malignancy. In the present study, livers from rats treated with DEN-CCl₄ alone in both the promotion and progression experiments had a significantly higher expression of PCNA than those treated with vehicle. After AND treatment, the PCNA expression was reduced in both experiments, suggesting that liver cell proliferation was regulated upon AND treatment.

AST and ALT are normally located in liver cells. When liver cells are injured, these two enzymes will leak out into the general circulation and cause an elevated level in blood serum. Hence, AST and ALT are often used as an indicator to evaluate the degree of liver damage. In the promotion and progression experiments, the serum levels of AST and ALT in AND-treated rats were significantly lower than those in the positive control rats (Fig. 15). The findings, therefore, suggest that liver damage was significantly reduced in rats that had been treated with AND in both the promotion and progression stages of hepatocarcinogenesis.

AND reduces the expression of GST-P foci

Immunohistochemical identification of GST-P has been used widely for the detection of preneoplastic lesion in rat hepatocarcinogenesis (104). GST-P is normally absent in rat hepatocytes but it can be induced by various xenobiotics. It has been shown that GST-P mRNA is induced by lead nitrate, aflatoxin metabolites or phenobarbital (105, 106). Several studies also reported that GST-P can be induced by epidermal growth factor (EGF) or insulin (107). In the present study, no GST-P foci could be detected in the negative control liver sections but clusters or groups of GST-P foci were extensively observed in the positive control sections (Fig. 18 & 19). The expression of GST-P foci in the DEN-CCl₄ treated livers indicates the induction of preneoplastic lesions in both promotion and progression stages of carcinogenesis. Upon treatment of AND at a dose of 10 mg/kg for 24 and 52 weeks, a decrease in the number and area of GST-P positive foci was detected at these two stages (Fig. 18 & 19). The results demonstrate that AND can reduce the expression of GST-P and thus the number of preneoplastic foci that were induced by DEN-CCl₄.

AND restores the morphology of normal liver

Gross examination of the rat liver that had been treated with DEN-CCl₄ showed the presence of lipid droplets, nodules and eventually tumors across the surface of the liver. There was also a significant increase in the relative liver weights in these rats as compared with those in the vehicle-treated rats. This phenomenon is typical in the treatment of CCl₄ to rats. The carcinogen induces CYP 2E1 which activates oxygen via an NADPH-dependent mechanism (108). Subsequent dissociation of superoxide radicals from the enzyme-substrate complex generates free radicals that react with microsomal membranes to induce lipid peroxidation that leads to cell

membrane damage (109). The chronic administration of certain xenobiotics and the metabolizing enzymes has been reported to induce liver enlargement in rats (110). The increase of the relative liver weight with CCl₄ is thought to be due to liver cell proliferation called hyperplasia (111). Consistent with this observation, significant hyperplasia could be detected in the livers of rats that had been treated with DEN-CCl₄ in both the promotion and progression experiments (Fig. 14). This induction of cell proliferation becomes particularly important for tumorigenesis in slowly proliferating tissues such as liver.

In the promotion experiment, the AND-treated livers retained a smooth surface and generally resembled the negative control livers (Fig. 16). It was observed that some nodules were present in the livers that had been treated with AND in the progression stage (Fig. 17). However, the structure of the liver generally resembled that of the normal livers. The results reflect that AND restores the basic structure of rat livers.

Moreover, histological examination of rat livers revealed that hepatocytes of AND- treated livers adopted the well-defined, cuboidal shape that is characteristic of normal hepatocytes. Swollen hepatocytes and cytoplasmic vacuolization, which are common in CCl₄ intoxication, were only observed in positive control sections in both experiments. The findings further indicate that AND can retain the normal hepatic morphology.

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WHAT IS CLAIMED IS:

1. A method for the inhibition of proliferation of cancer cells, or the induction of apoptosis of cancer cells, comprising contacting the cancer cells with an effective amount of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier.
2. The method of claim 1, wherein said cancer cells are liver cancer cells, preferably human liver cancer cells.
3. The method of claim 1 or 2, wherein said inhibition of proliferation of cancer cells comprises an accumulation of the cells in G₀/G₁ phase of the cell cycle.
4. The method of claim 1 or 2, wherein said AND or composition down-regulates expression of Bcl-2 or Bcl-w protein, and/or up-regulates expression of Bax protein, and/or decreases expression of Mdm2 protein and/or increases stability of p53 protein.
5. A method for preventing, treating and/or reducing liver damage, or preventing or treating liver cancer in a subject, comprising administering the subject a therapeutically effective amount of AND or a composition comprising AND and a pharmaceutically acceptable carrier.
6. The method of claim 5, wherein said liver comprises a mammal liver, preferably a human liver.
7. The method of claim 5 or 6, wherein said liver damage is induced by an agent selected from the group consisting of DEN and CCl₄, nitrate, aflatoxin metabolites and phenobarbital.
8. The method of any one of claims 5 to 7, wherein said liver damage comprises a preneoplastic lesion in hepatocarcinogenesis.

9. A method for the prevention and/or treatment of liver cancer in a subject comprising administering the subject a therapeutically effective amount of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier.

10. The method of claim 9, wherein said liver cancer comprises hepatocellular carcinoma, a cholangiocarcinoma, or a cholangiocellular carcinoma.

11. The method of claim 9, wherein said subject is an animal (e.g., birds, reptiles, and mammals), preferably a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, cat, dog, rat, and mouse) and a primate (e.g., a monkey, chimpanzee, and a human).

12. Use of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier for the inhibition of proliferation of cancer cells, the induction of apoptosis of cancer cells, the prevention, treatment and/or reduction of liver damage, or the prevention and/or treatment of liver cancer in a subject.

13. Use of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for the inhibition of proliferation of cancer cells, or the induction of apoptosis of cancer cells in a subject.

14. The use of claim 12 or 13, wherein said cancer cells are liver cancer cells, preferably human liver cancer cells.

15. The use of any one of claims 12 to 14, wherein said inhibition of proliferation of cancer cells comprises an accumulation of the cells in G₀/G₁ phase of the cell cycle.

16. The use of any one of claims 12 to 14, wherein said AND or composition down-regulates expression of Bcl-2 or Bcl-w protein, and/or up-regulates expression of Bax protein, and/or decreases expression of Mdm2 protein and/or increases stability of p53 protein in the cancer cells.

17. The use of any one of claims 12 to 16, wherein said subject is an animal (e.g., birds, reptiles, and mammals), preferably a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, cat, dog, rat, and mouse) and a primate (e.g., a monkey, chimpanzee, and a human).

18. Use of andrographolide (AND) or a composition comprising AND and a pharmaceutically acceptable carrier in preparing a medicament for the prevention and/or treatment of liver cancer, or the prevention, treatment and/or reduction of liver damage in a subject.

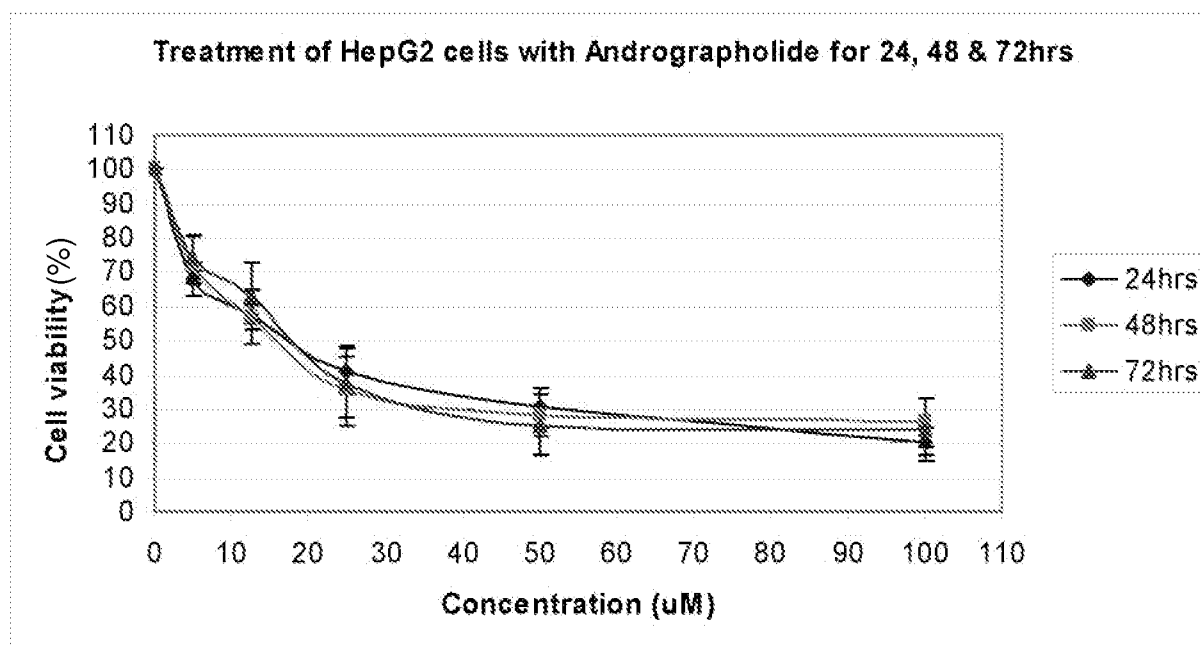
19. The use of claim 12 or 18, wherein said liver damage is induced by an agent selected from the group consisting of DEN and CCl₄, nitrate, aflatoxin metabolites and phenobarbital.

20. The use of any one of claims 12, 18 and 19, wherein said liver damage comprises a preneoplastic lesion in hepatocarcinogenesis.

21. The use of claim 12 or 18, wherein said liver cancer comprises hepatocellular carcinoma, a cholangiocarcinoma, or a cholangiocellular carcinoma.

22. The use of any one of claims 18 to 21, wherein said subject is an animal (e.g., birds, reptiles, and mammals), preferably a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, cat, dog, rat, and mouse) and a primate (e.g., a monkey, chimpanzee, and a human).

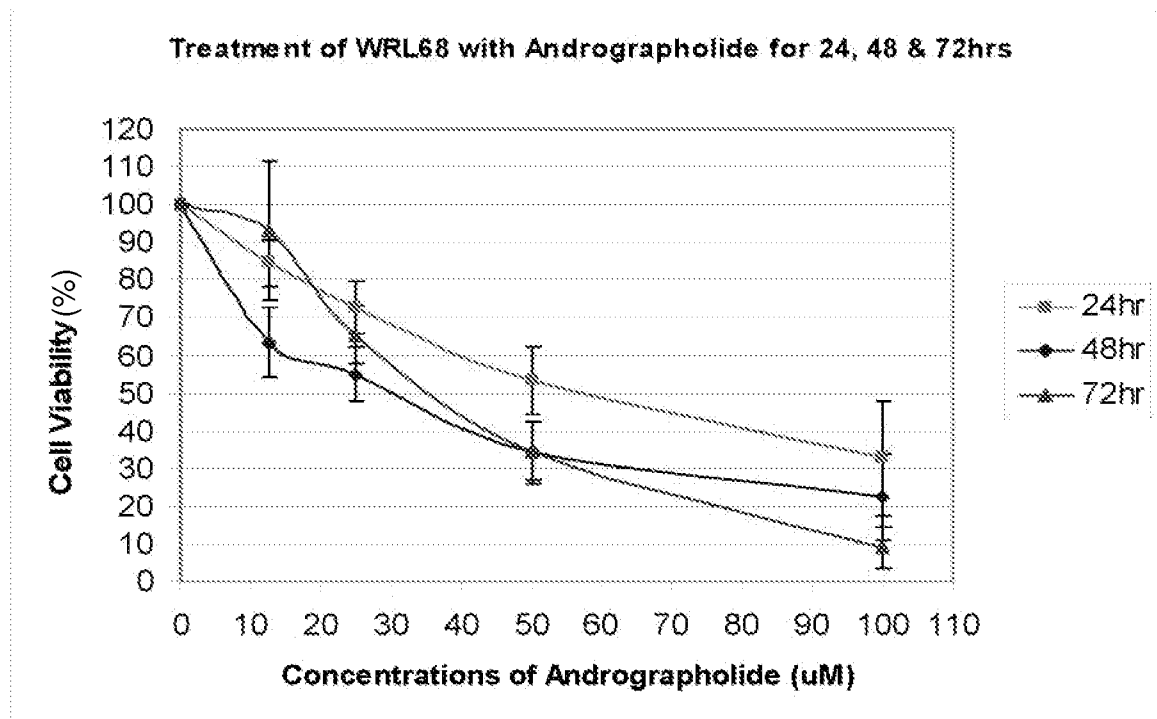
Cell viability of HepG2 treated with AND for 24, 48 & 72 hrs.



Time (hrs)	24	48	72
IC ₅₀ (μM)	17.5	16	17

Figure 1

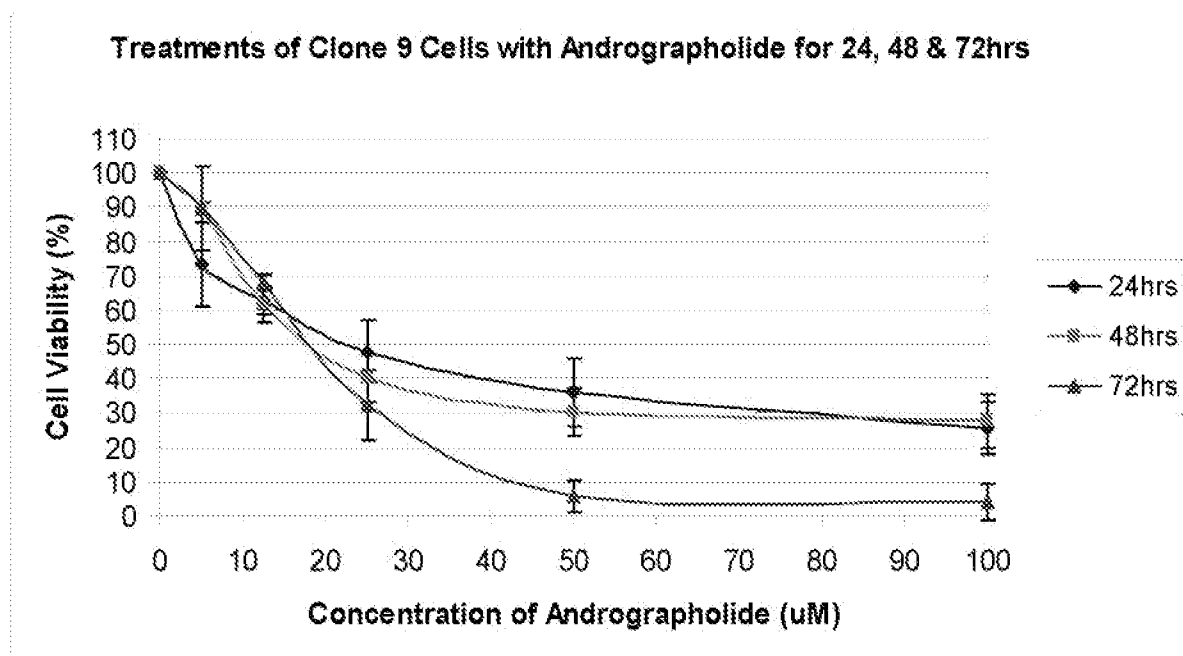
Cell viability of WRL 68 treated with AND for 24, 48 & 72 hrs.



Time (hrs)	24	48	72
IC ₅₀ (uM)	57	30	35

Figure 2

Cell viability of Clone 9 treated with AND for 24, 48 & 72 hrs.



Time (hrs)	24	48	72
IC ₅₀ (μM)	22	18	18

Figure 3

DNA profiles of HepG2 cells treated with DMSO and AND for 24 hrs

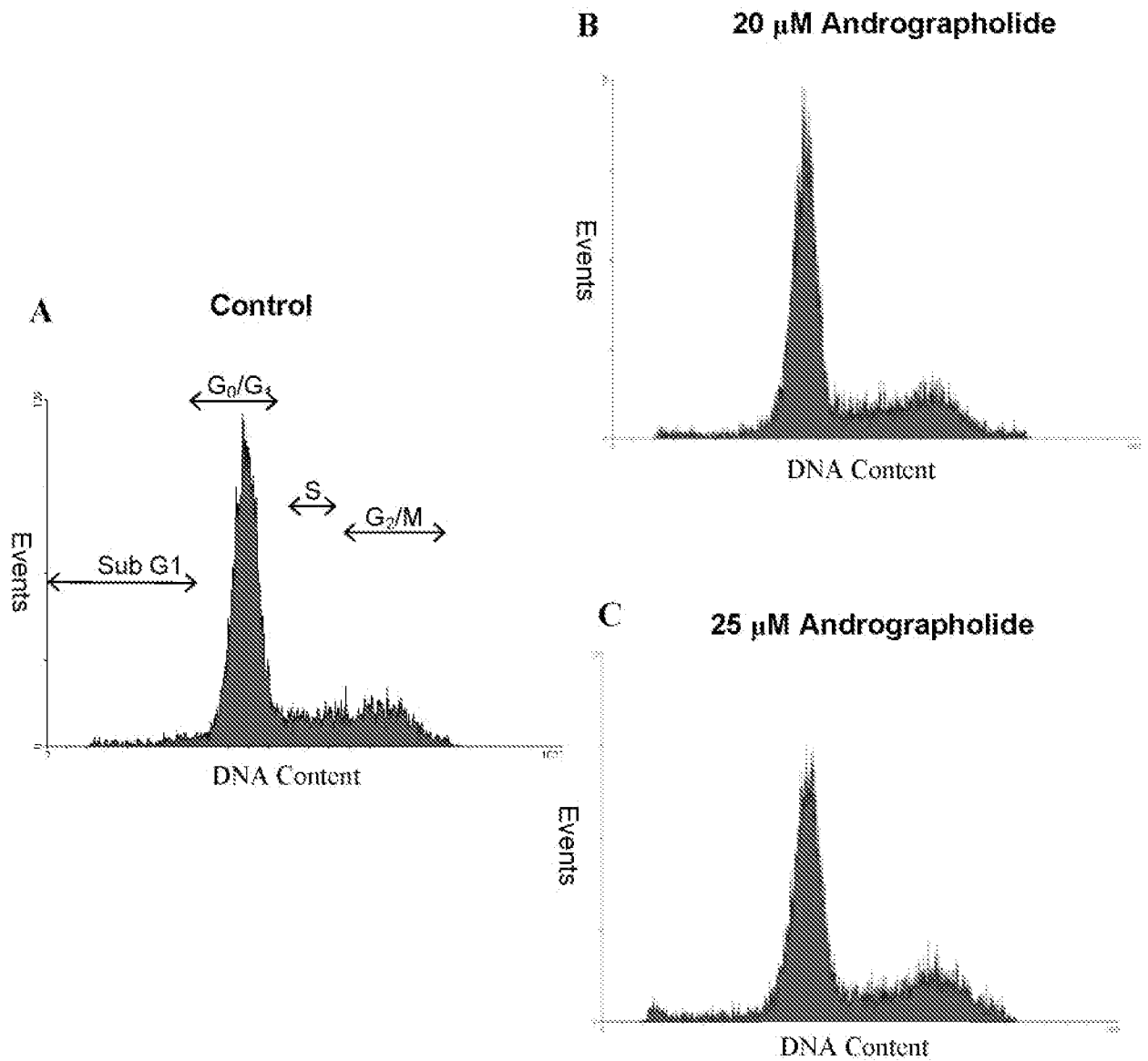
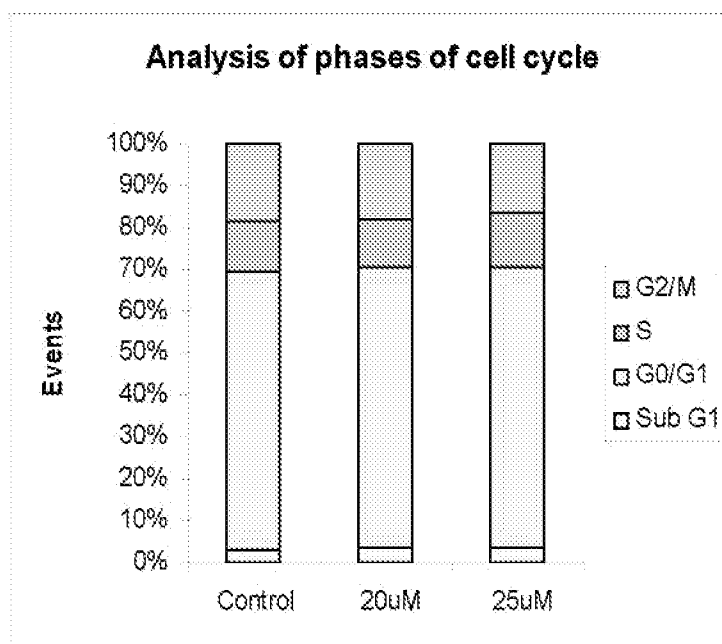


Figure 4

**Analysis of the cell cycle upon treatment of HepG2 with DMSO and AND
for 24 hrs**



Sample	Sub G ₁	G ₀ /G ₁	S	G ₂ /M
Control	2.9% ± 0.3	66.7% ± 1.7	12.3% ± 1.5	18.4% ± 1.4
20 μM	3.3% ± 0.4	67.4% ± 1.3	11.3% ± 0.8	18.3% ± 1.1
25 μM	3.4% ± 0.2	66.3% ± 1.6	14.0% ± 0.6	16.7% ± 1.0

Figure 5

DNA profiles of HepG2 cells treated with DMSO and AND for 48 hrs

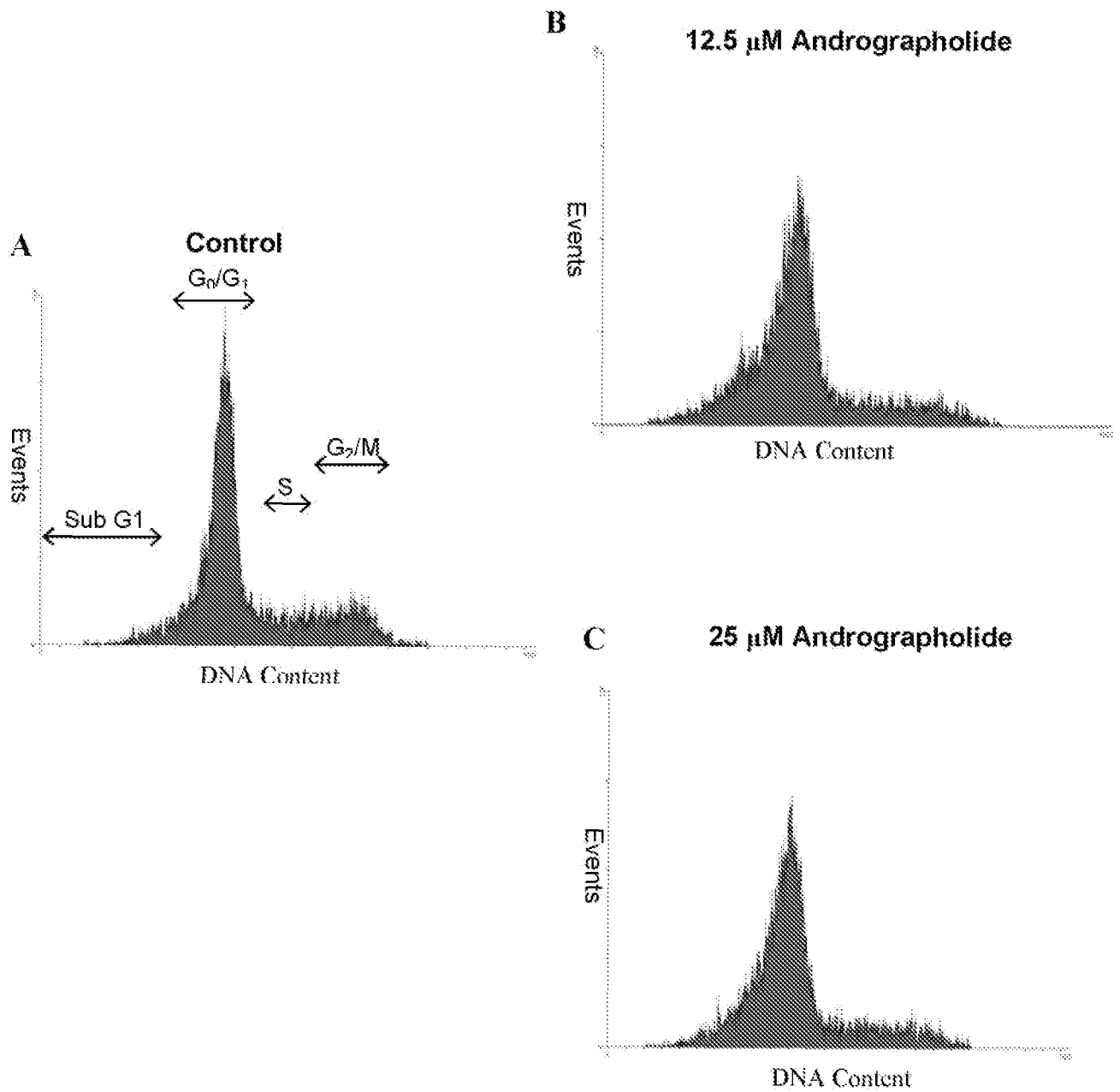
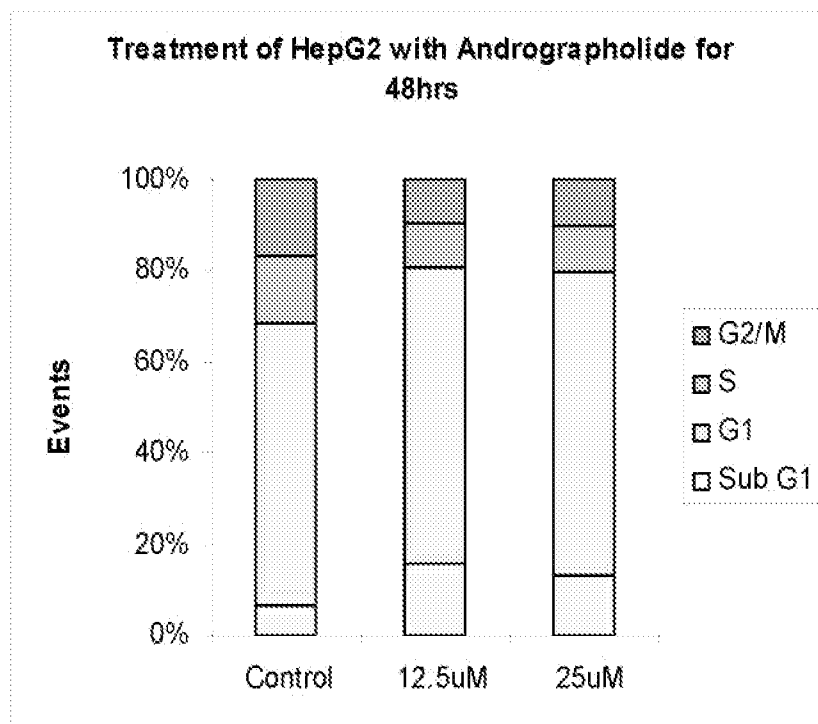


Figure 6

Analysis of the cell cycle upon treatment of HepG2 with DMSO and AND for 48 hrs



Sample	Sub G ₁	G ₀ /G ₁	S	G ₂ /M
Control	6.1% ± 0.5	62.1% ± 0.8	15.1% ± 0.8	16.7% ± 1.5
12.5 μM	14.5% ± 0.8	65.4% ± 0.6	9.7% ± 0.5	9.8% ± 1.1
25 μM	13.1% ± 0.6	67.1% ± 0.8	9.7% ± 0.7	10.4% ± 0.7

Figure 7

Fragmentation of DNA in HepG2 cells after AND
treatment

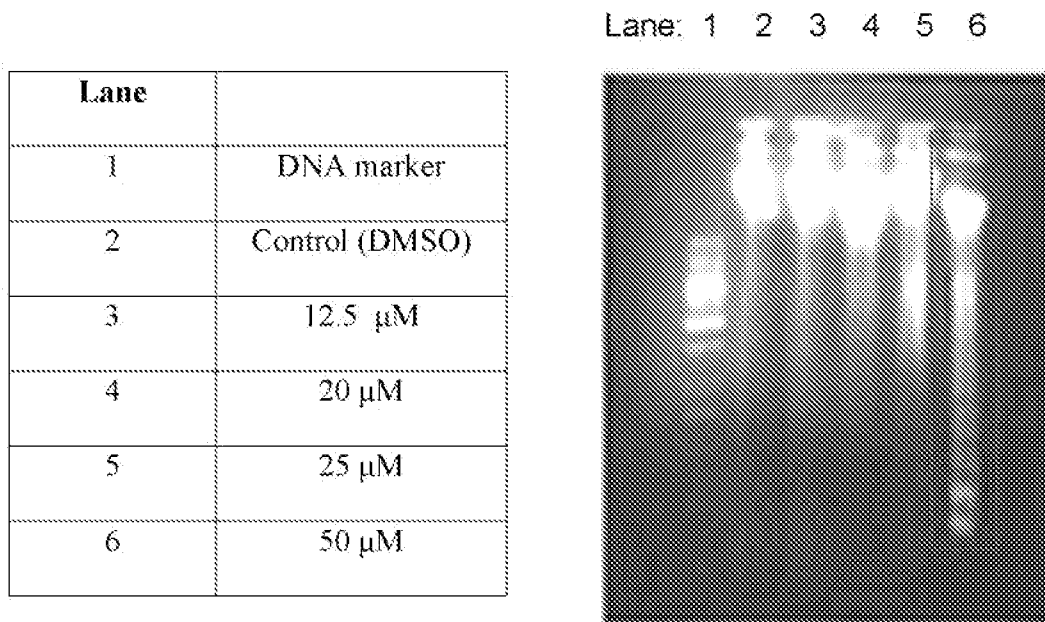


Figure 8

The scatter plot of gene expressions from HepG2 cells treated with DMSO
and AND

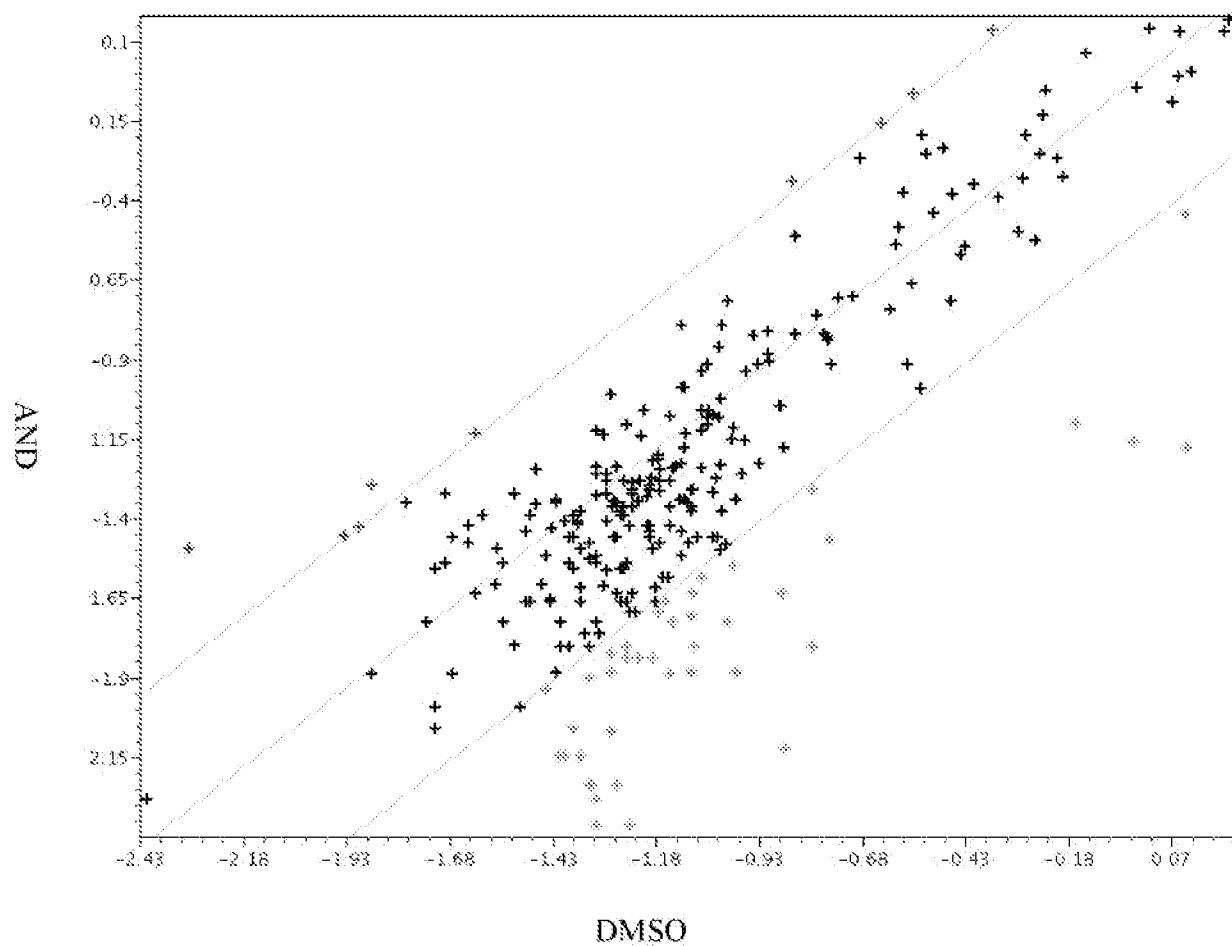
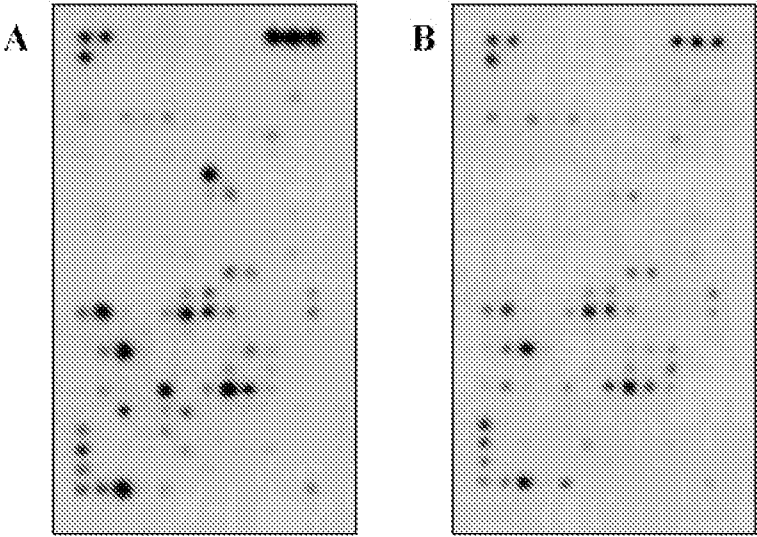


Figure 9

cDNA microarray analysis



Symbol	Gene name	Fold changes
BCL2	Bcl-2	-4.9
BCL2L2	Bcl-w	-3.2
CCND1	Cyclin D1	-3.6
CHEK2	CHK2	-4.3
MDM2	Mdm2	-3.4
NF-κB2	NF-κB2	-3.1
RB1	Rb	+3.2
SOD1	Cu/ZnSOD	+3.1

Figure 10

Effects of diethylnitrosamine (DEN) and carbon tetrachloride (CCl₄) on
rat liver

A



B

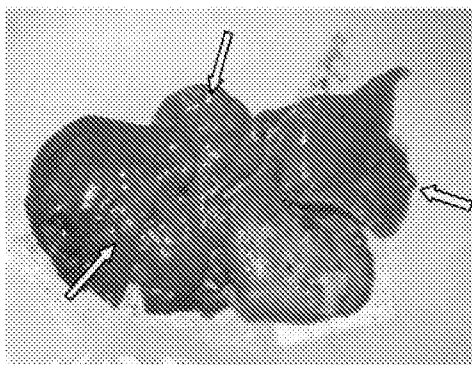


Figure 11

Gross examination of rat livers in the promotion stage of
hepatocarcinogenesis

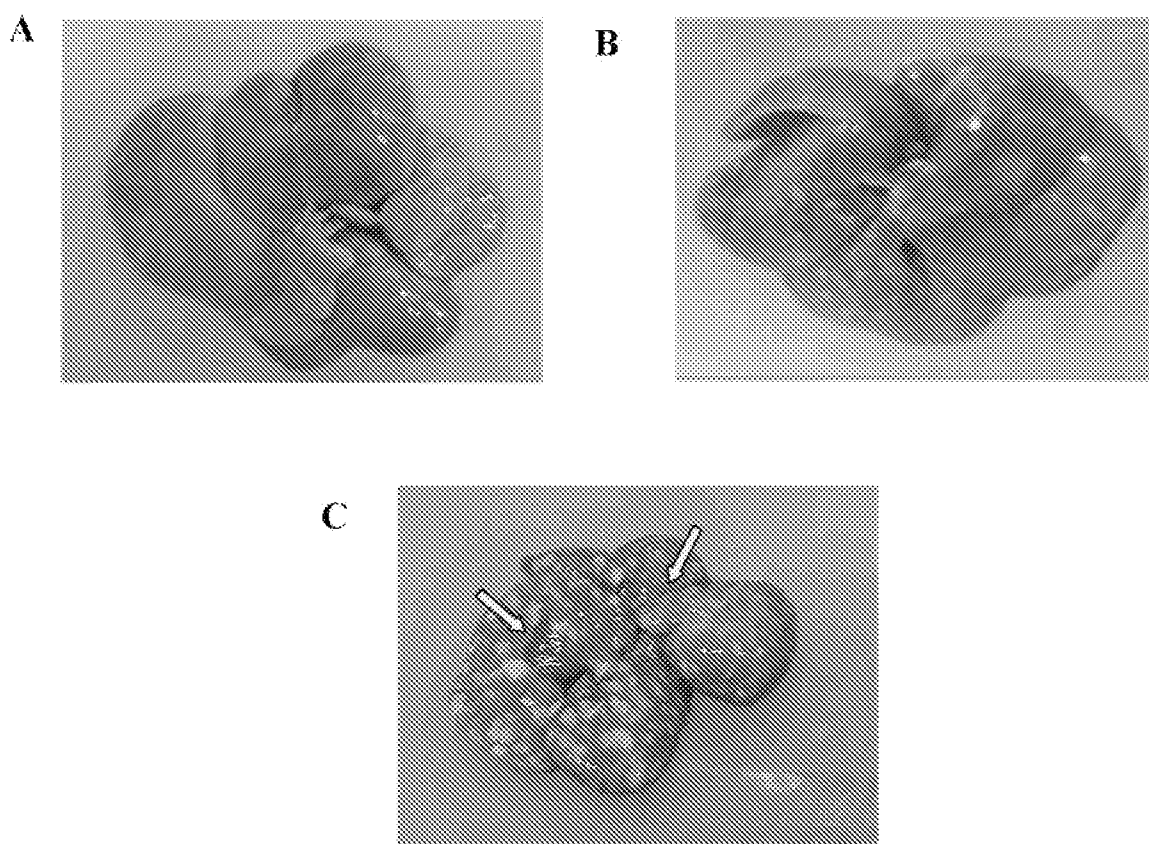


Figure 12

Gross examination of rat livers in the progression stage of
hepatocarcinogenesis

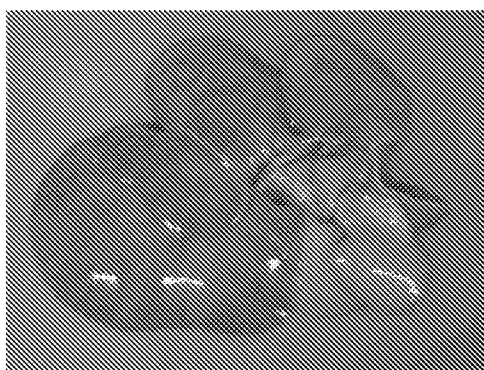
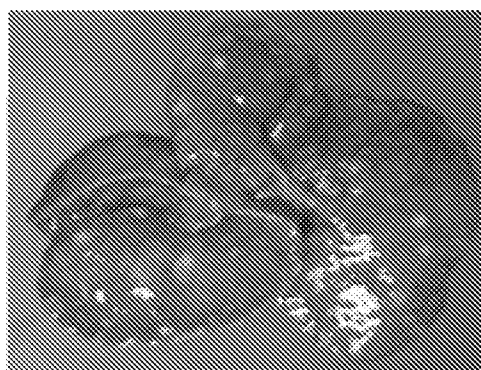
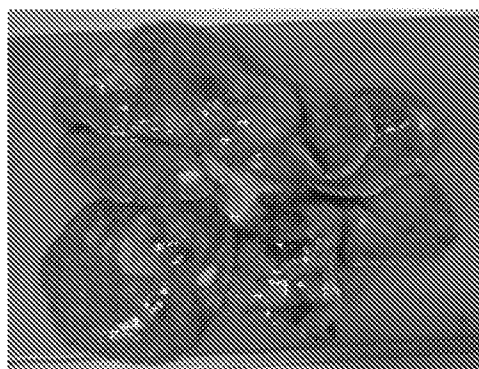
A**B****C**

Figure 13

Effects of AND on the liver weight of rats in promotion and progression groups

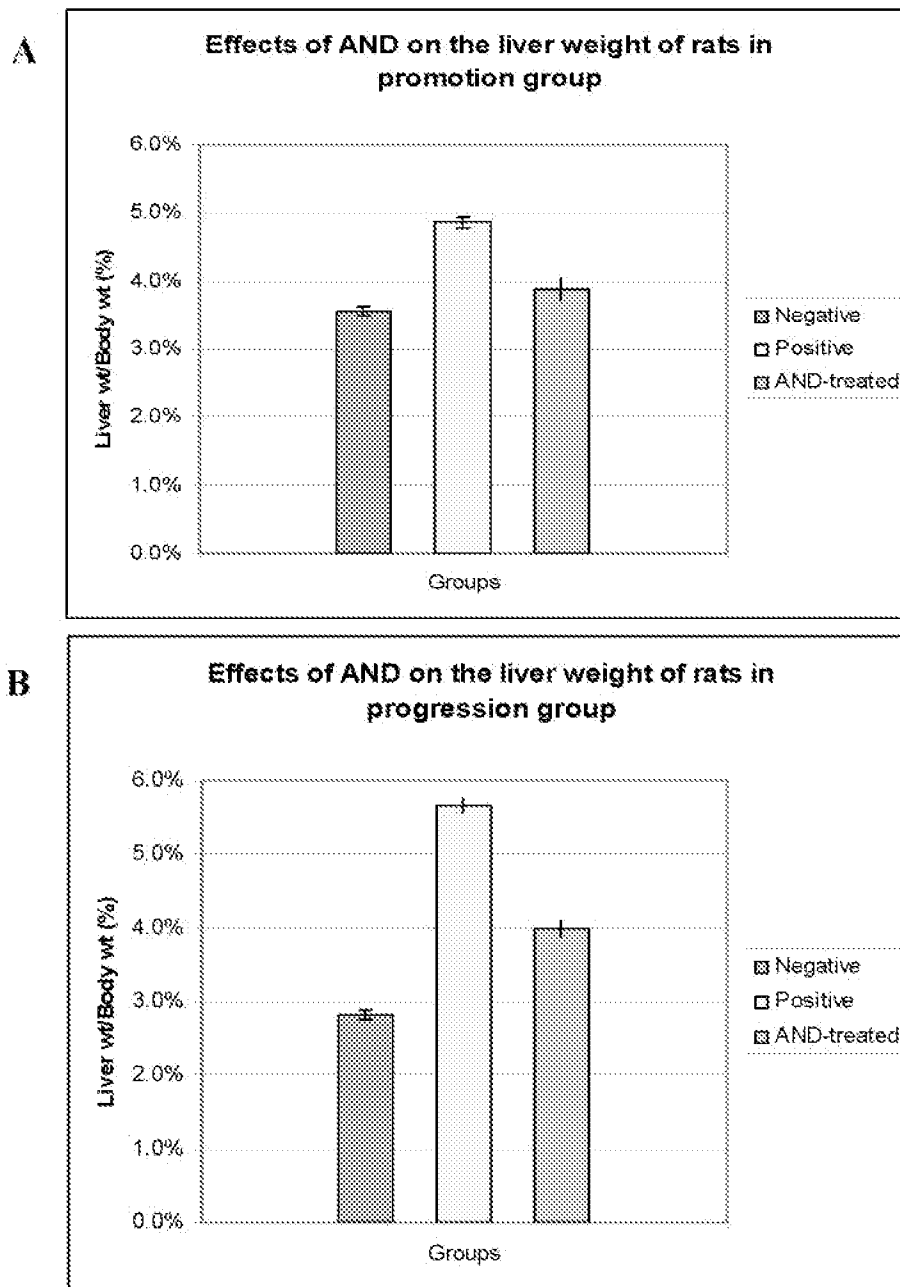
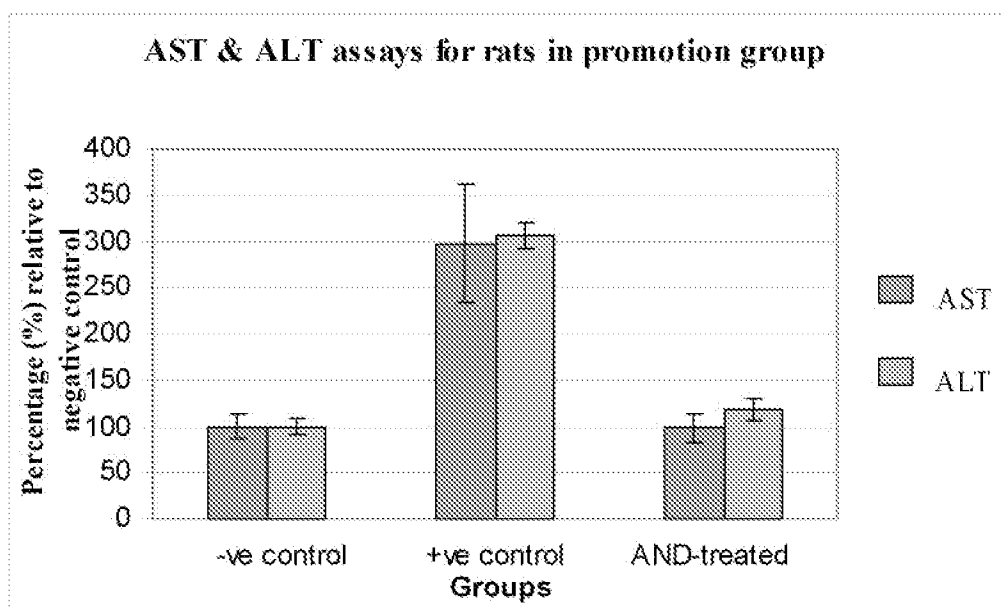
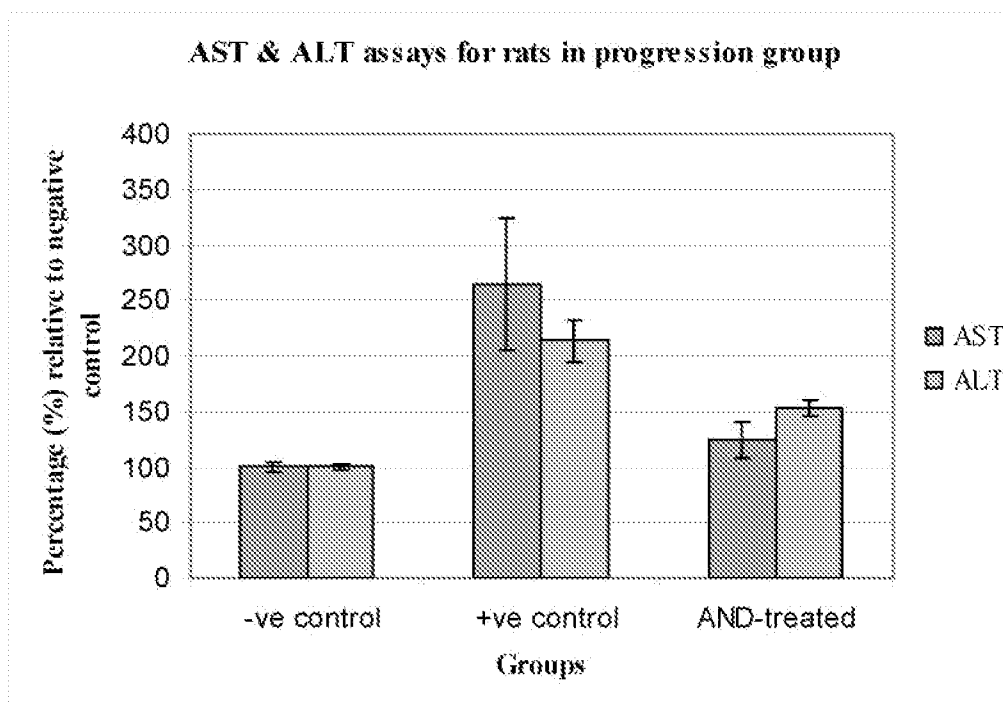


Figure 14

AST and ALT assays for the promotion and progression groups of rats**A****B****Figure 15**

Hematoxylin & Eosin (H&E) staining of rat livers in the promotion stage
of hepatocarcinogenesis

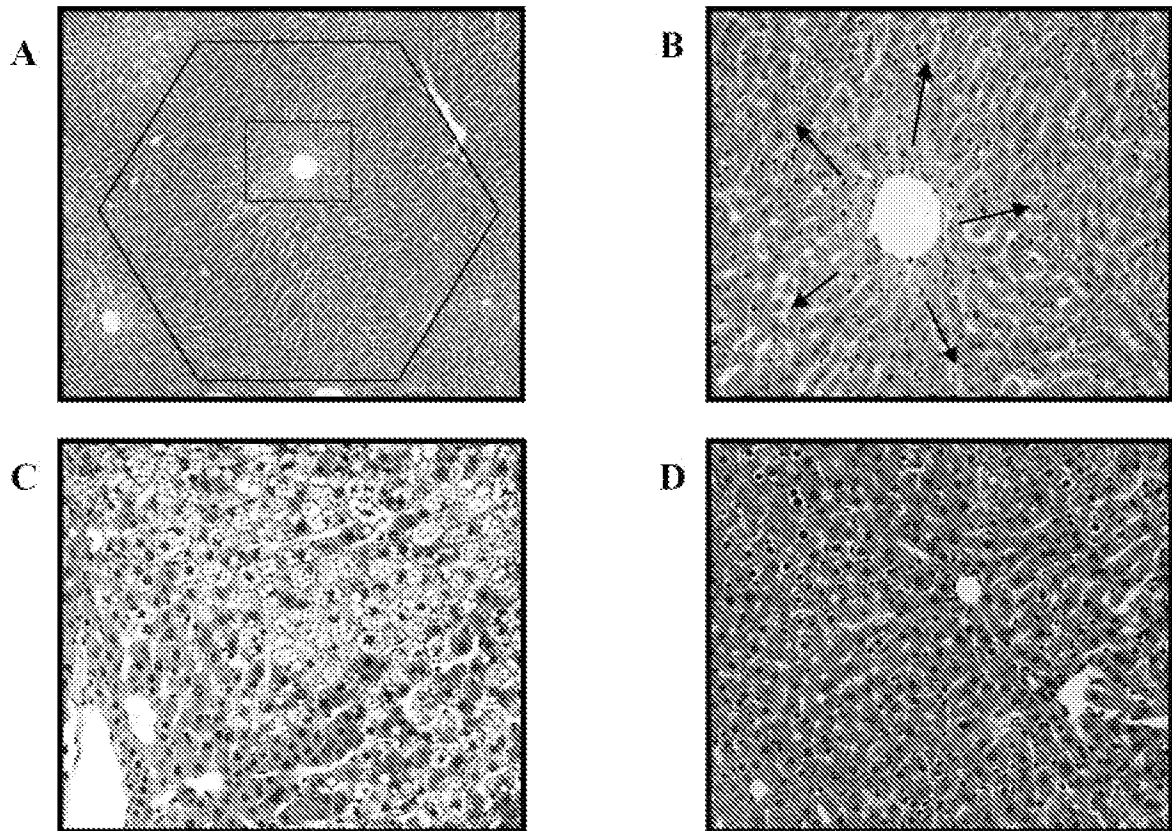


Figure 16

Hematoxylin & Eosin (H&E) staining of rat livers in the progression
stage of hepatocarcinogenesis

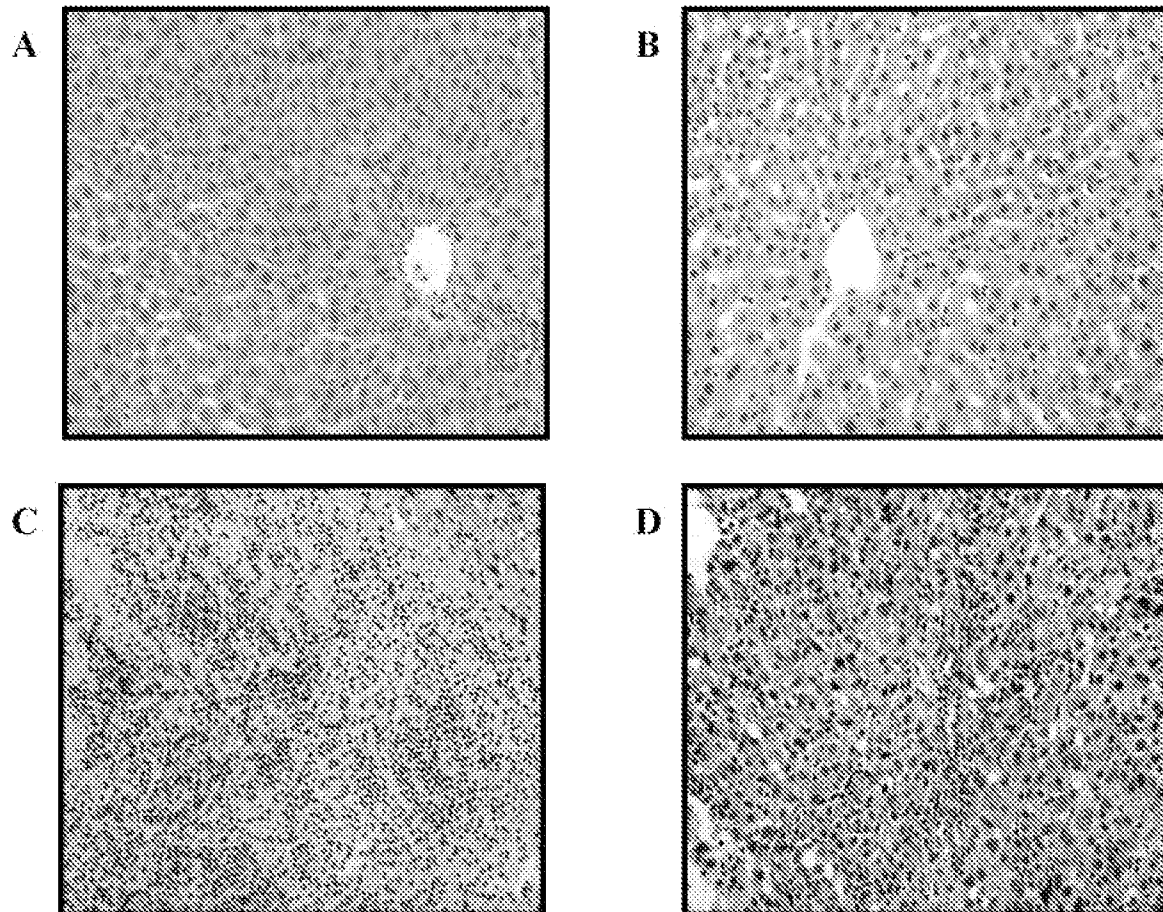


Figure 17

Immunostaining of GST-P of rat liver sections in the promotion group

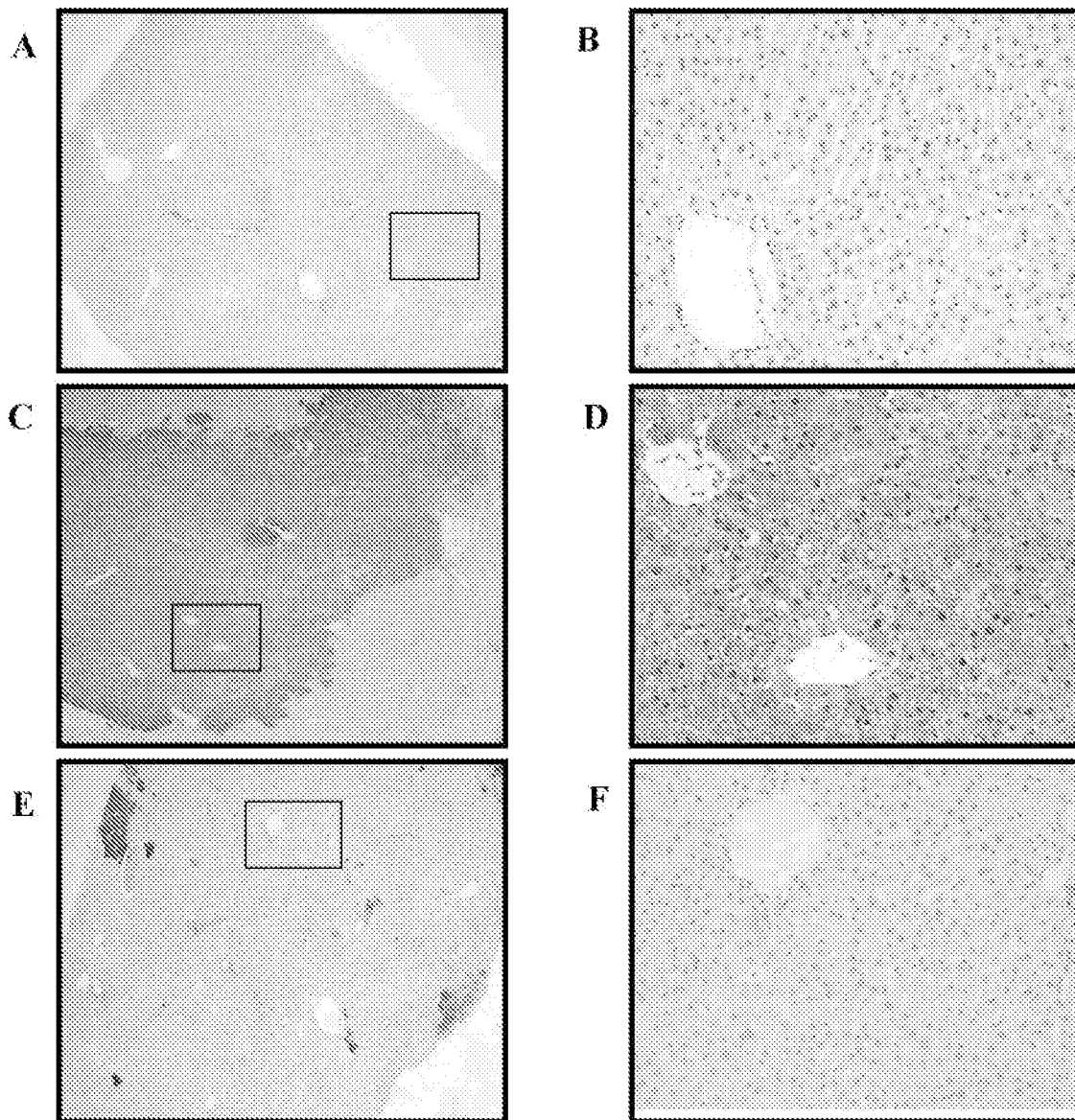


Figure 18

Immunostaining of GST-P of rat liver sections in the progression group

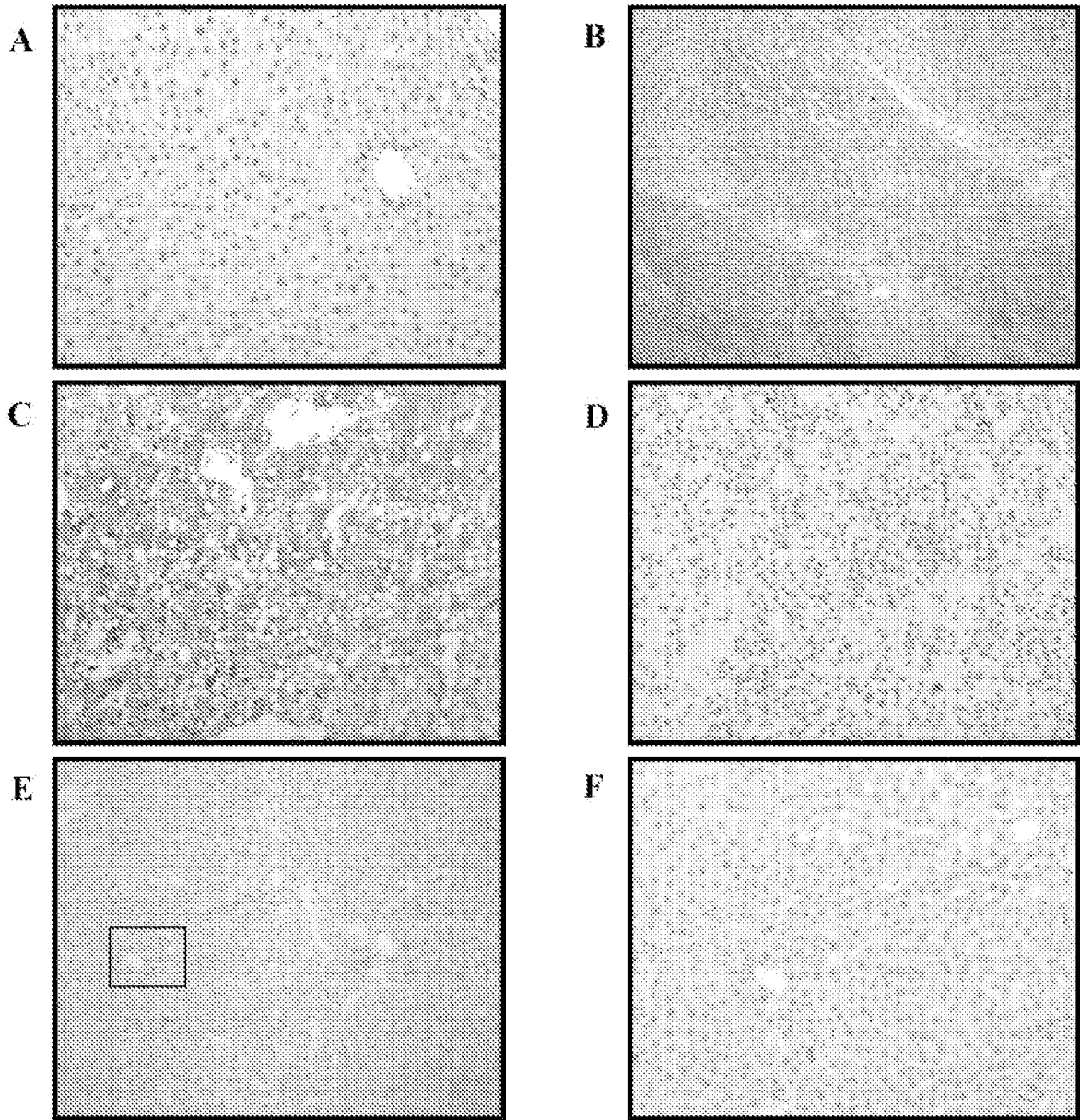


Figure 19

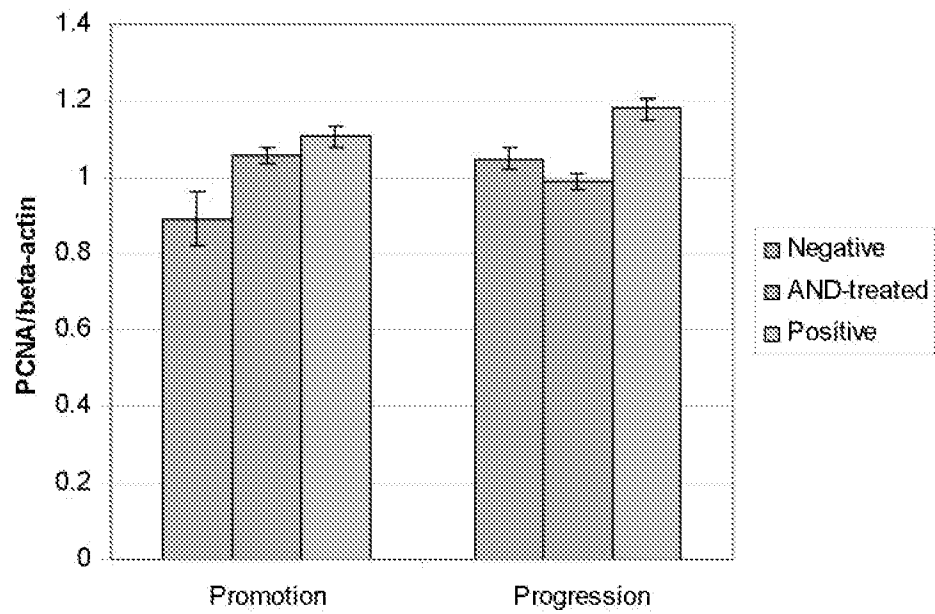
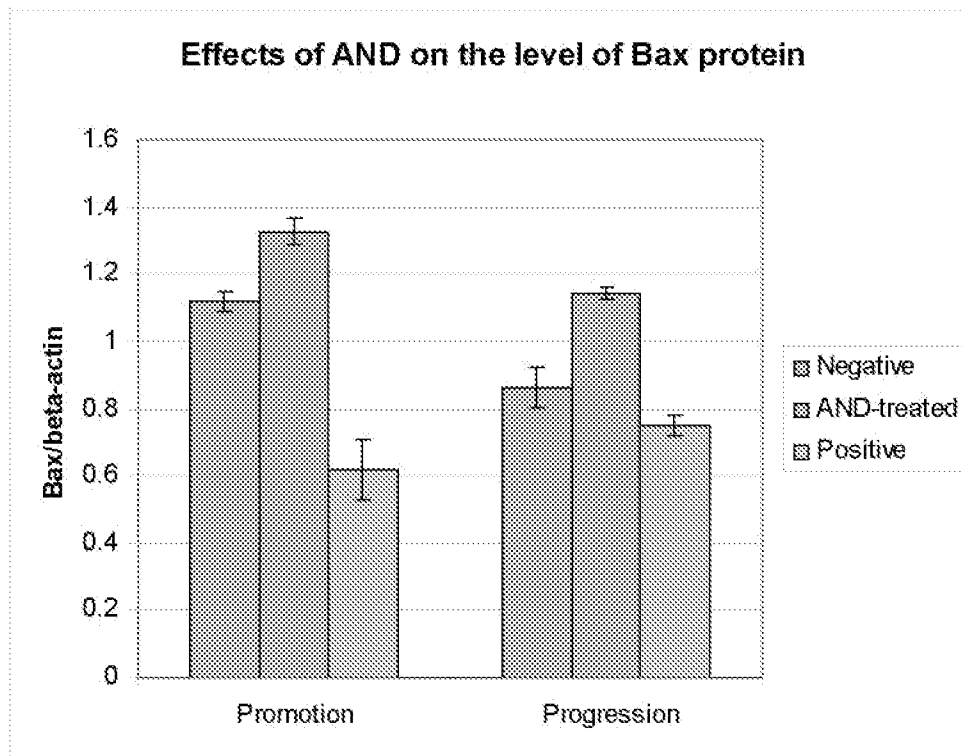
Western blot analysis of PCNA protein**A****Effects of AND on the level of PCNA protein****B**PCNA
 β -actin

Figure 20

Western blot analysis of Bax protein**A****B**

Bax
 β -actin

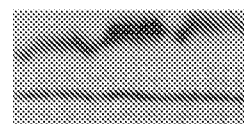


Figure 21

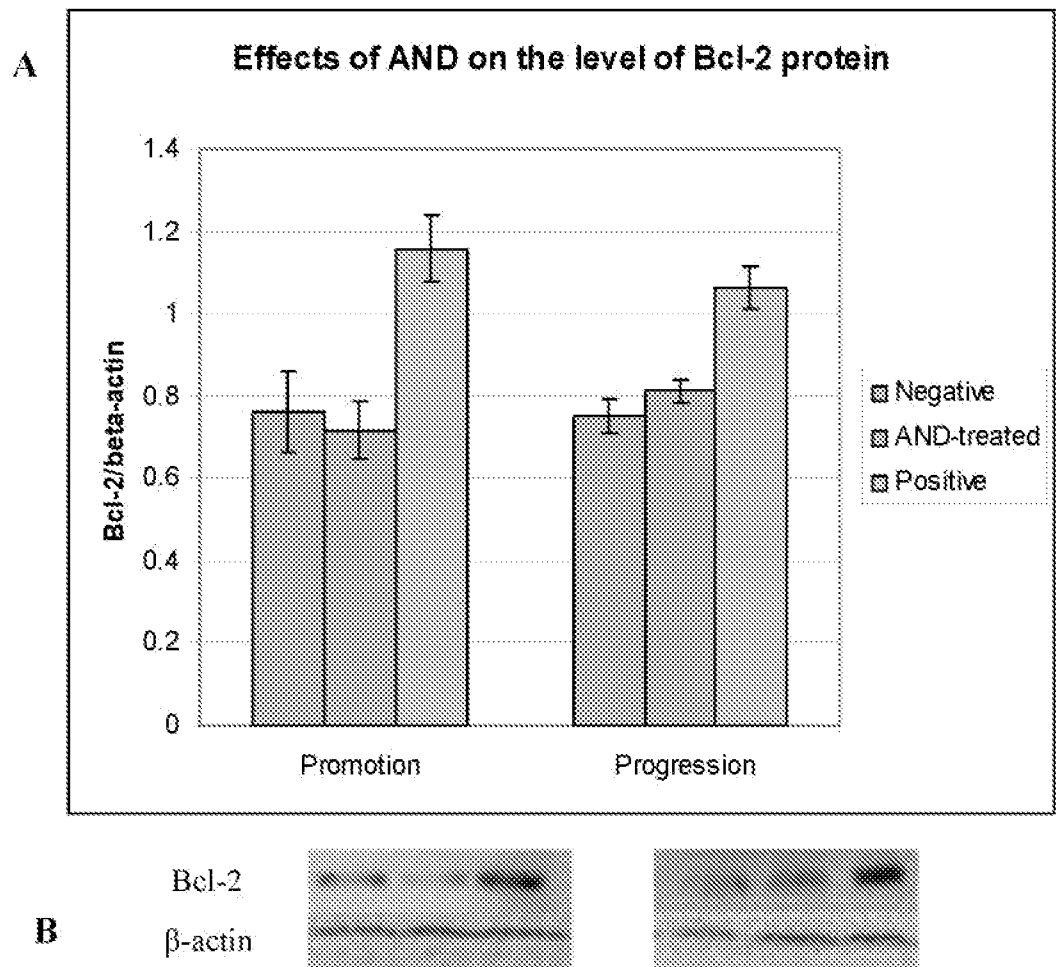
Western blot analysis of Bcl-2 protein

Figure 22

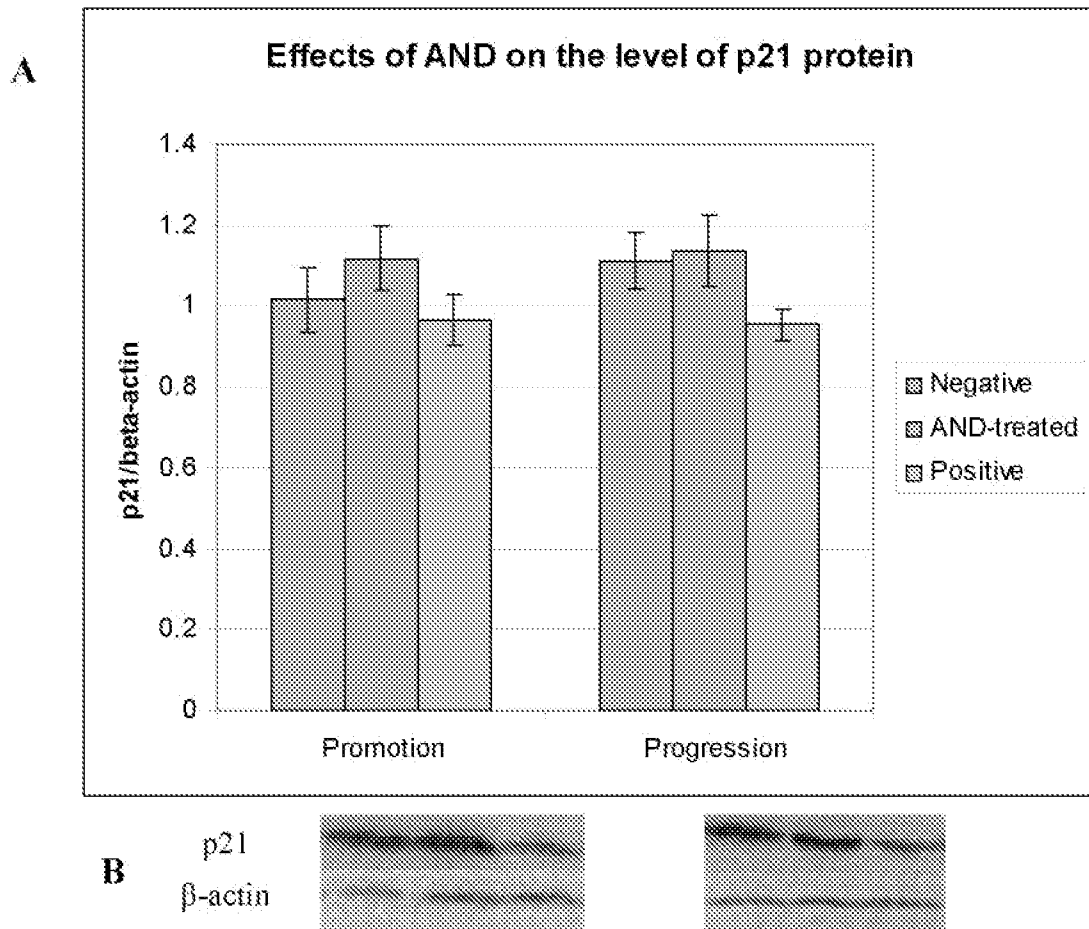
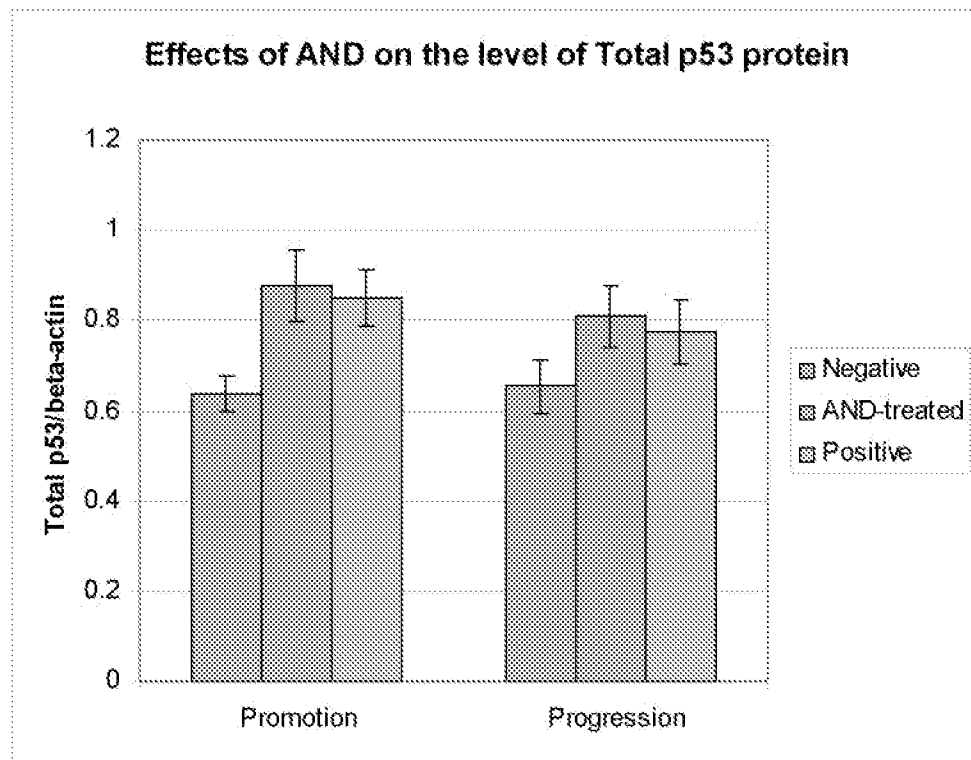
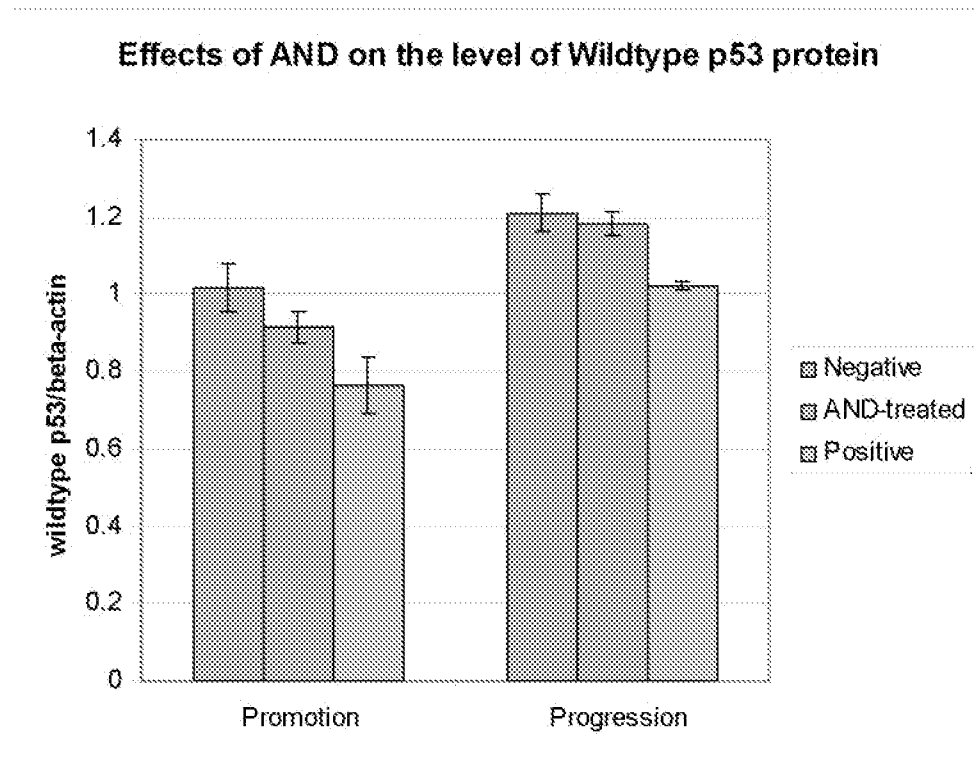
Western blot analysis of p21 protein

Figure 23

Western blot analysis of total p53 protein**A****B**

Total p53
 β -actin

**Figure 24**

Western blot analysis of wildtype p53 protein**A****B**

WT p53
 β -actin

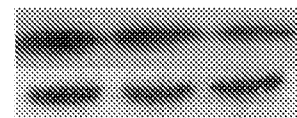
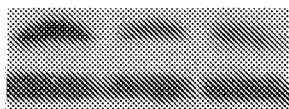


Figure 25

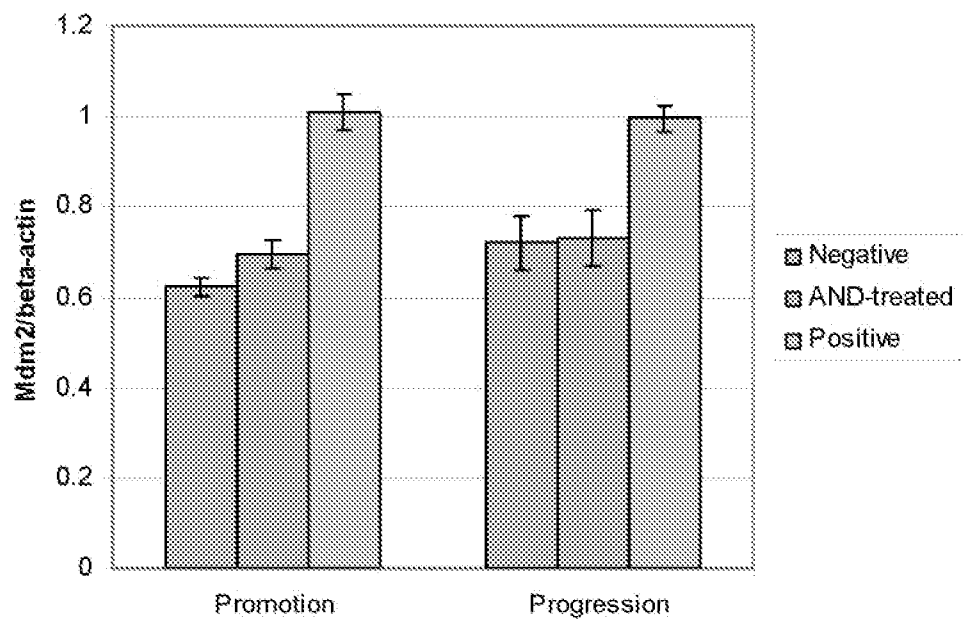
Western blot analysis of Mdm2 protein**A****Effects of AND on the level of Mdm2 protein**

Figure 26

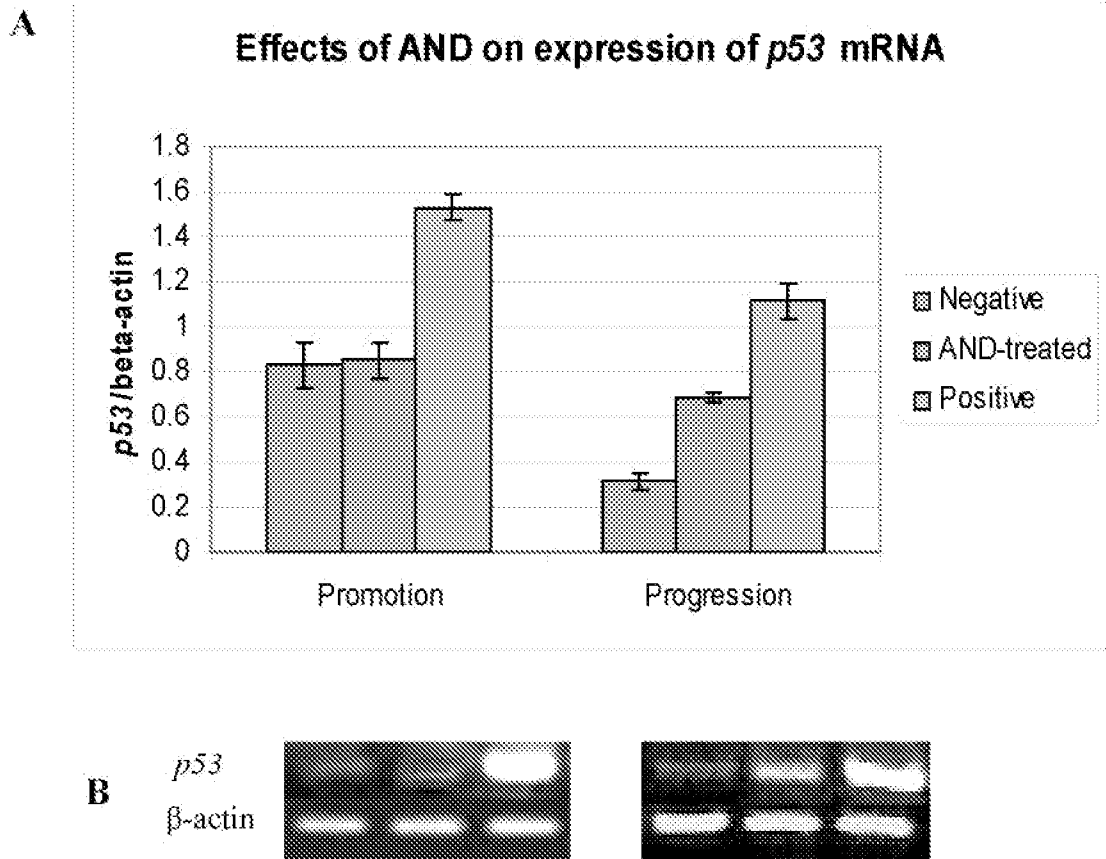
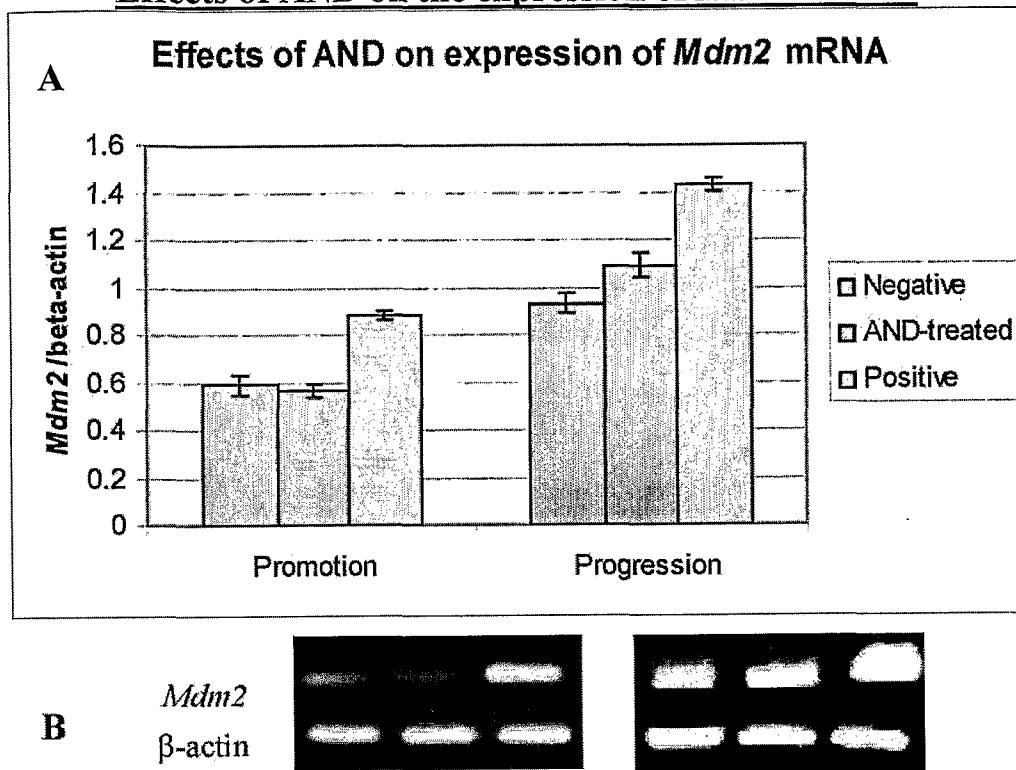
Effects of AND on the expression of *p53* mRNA

Figure 27

Effects of AND on the expression of *Mdm2* mRNA**Figure 28**

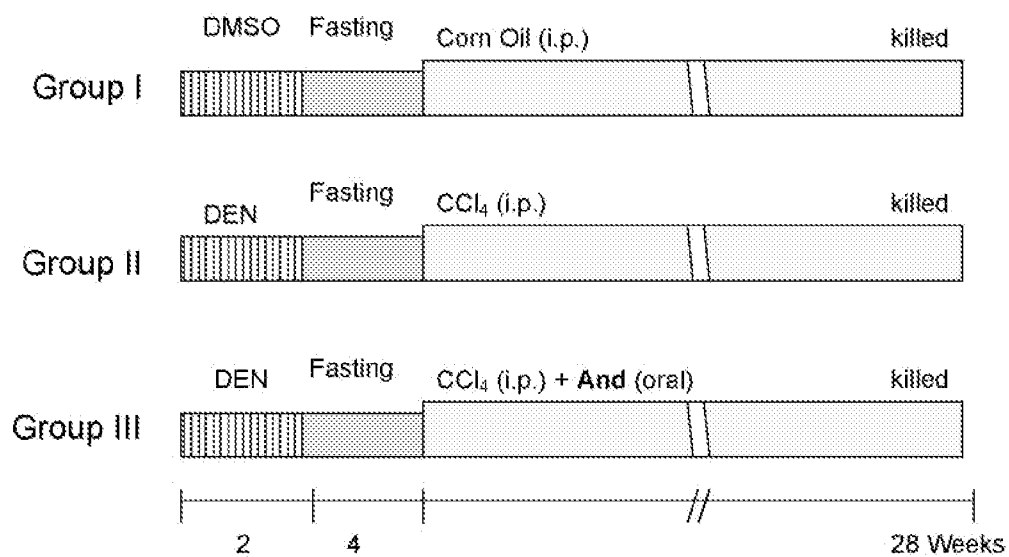
Experimental protocol for rat treatments in Experiment 1

Figure 29

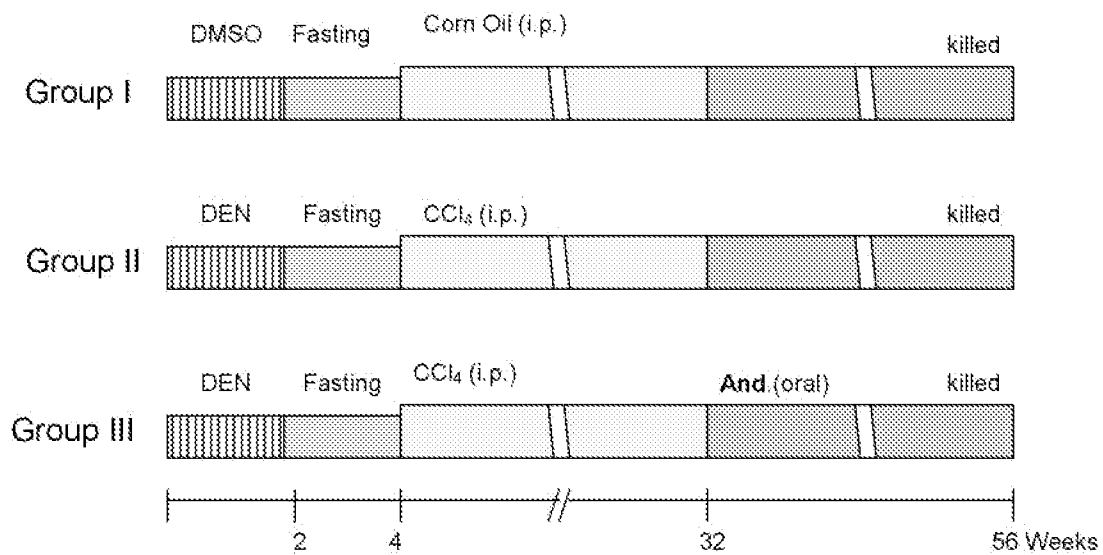
Experimental protocol for rat treatments in Experiment II

Figure 30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2009/072693

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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, A61P

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)

CNPAT(Cprs), CTCMPD, CNKI Full-Text database, Chinese Medicine Abstract, WPI, EPODOC, PAJ, CA, Embase,;

Andrographolide, cancer, tumor, liver cancer, hepato-, carcinogenesis, hepatocellular carcinoma, cholangiocarcinoma, cholangiocellular,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Qi Cuiling et al. Advances in study on anti-tumor mechanism of andrographolide. China Journal of Chinese Materia Medica(Chinese). Oct. 2007, Vol. 32 Issue 20, pages 2095-2097, ISSN1001-5302	1-6, 9-18, 21-22
X	Liu Guoli et al. Advances in study on andrographolide. Herald of Medicine(Chinese). Jan. 2006, Vol. 25 No. 1, pages 48-50, ISSN1004-0781	7, 8, 19, 20

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

* Special categories of cited documents:	“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
“A” document defining the general state of the art which is not considered to be of particular relevance	“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
“E” earlier application or patent but published on or after the international filing date	“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
“L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)	“&” document member of the same patent family
“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	

Date of the actual completion of the international search
10 Sep. 2009(10. 09. 2009)Date of mailing of the international search report
01 Oct. 2009 (01.10.2009)Name and mailing address of the ISA/CN
The State Intellectual Property Office, the P.R.China
6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China
100088
Facsimile No. 86-10-62019451

Authorized officer

CHENYANYAN

Telephone No. (86-10)62411200

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2009/072693

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.: 1-12, 14-17, 19-22
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-12, 14-17, 19-22 are directed to a method or use of treatment of the human/animal body(Article 17 (2) (a) (i) and Rule 39. 1(iv) PCT). However, the search has been carried out and based on the alleged effects of the pharmaceutical compositions and the use in manufacture of medicaments in claims 1-12, 14-17, 19-22.
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 fees, this Authority did not invite payment of 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

International application No.

PCT/CN2009/072693

CLASSIFICATION OF SUBJECT MATTER

A61K 31/365(2006. 01)i

A61P 35/04(2006. 01)i