Antitumoral effect of Ocoxin in hepatocellular carcinoma

ELENA DÍAZ-RODRÍGUEZ1, AL-MAHY EL-MALLAH1,3, EDUARDO SANZ2 and ATANASIO PANDIELLA1

1Institute of Molecular and Cellular Cancer Biology, CSIC-University of Salamanca, 37007 Salamanca; 2Catalysis, S.L., 28016 Madrid, Spain

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Abstract. Hepatocellular carcinoma (HCC) is becoming one of the most prevalent types of cancer worldwide. The most efficient types of treatment at present include surgical resection and liver transplantation, but these treatments may only be used in a small percentage of patients. In order to identify novel therapeutic strategies for this disease, the present study explored the potential antitumoral effect of Ocoxin® oral solution (OOS) in HCC. OOS inhibited the proliferation of HCC cell lines in a time- and dose-dependent manner, being more efficient when used in combination with sorafenib, a standard of care treatment for patients diagnosed with advanced-stage disease. Mechanistic studies indicated that the effect of OOS was due to the induction of cell cycle arrest rather than the stimulation of apoptotic cell death. The cell cycle was slowed down in all phases in the HCC cell lines treated with OOS. Finally, when tested in animal models of HCC, OOS reduced tumor progression through the induction of necrosis in xenograft tumor models. Considering the poor prognosis and high resistance to antitumor treatments of HCC, the antiproliferative action of OOS, particularly in combination with sorafenib, provides the opportunity to investigate the effect of combined therapy in a clinical setting.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is rapidly becoming one of the most prevalent types of cancer, being the sixth most common type of neoplasm worldwide and the third leading cause of cancer-associated mortality, responsible for more than 650,000 mortalities per year globally (1). The most efficient treatments for this disease include complete surgical resection of the tumor and liver transplantation. The outcome of HCC is typically poor since only 10-20% of HCCs may be completely removed by surgery and the lack of complete removal leads to the relapse of the disease (2).

HCC is a tumor characterized by being highly resistant to systemic chemotherapy. The use of the kinase inhibitor sorafenib has previously increased the overall survival of patients suffering from this disease (3-6). This agent, which may be orally administered, has antiproliferative and antiangiogenic activity and delays tumor progression, being at present the standard of care in patients diagnosed with an advanced-stage disease (7). However, the active search for other treatments that may be used to treat this malignancy is underway. Thus, during previous years, several phase III trials, including patients with intermediate and advanced-stages, have been carried out to test the effects of various drugs; however, in general, they have failed to present survival benefits (5), driving the search for the identification of novel oncogenic drivers and molecular therapeutics that may be used to treat HCC. In the previous decade, molecular analyses of patients with HCC have resulted in the identification of several genomic subclasses (5), although, thus far, there is no clear consensus with respect to the association between molecular subclass and patient outcome.

Several precedents indicate that natural products may be useful anticancer supplements. Numerous studies have indicated that green tea-derived polyphenol epigallocatechin-3-gallate exerts chemopreventive and hepatoprotective effects against HCC in preclinical models (8-10). The mechanism of action of this product has not been fully elucidated, but may include the reduction of prostaglandin biosynthesis by HCC cells (11), which may then induce apoptotic cell death in HCC. In addition, epigallocatechin-3-gallate may reduce the metastatic ability of HCC cells by decreasing the production of osteopontin (12).

Ocoxin® oral solution (OOS) is an oral nutritional supplement that contains several compounds with anticancer activities, including epigallocatechin-3-gallate (13). In addition, OOS is comprised of a variety of other components, including vitamins B6 and C and cinnamic acid, which exhibit anticancer properties (14-16). In addition, OOS contains glycyrrhizinic acid, which exhibits anti-inflammatory and immunomodulatory effects (17). As OOS contains several compounds with an antitumor action, it is currently being investigated in clinical trials as part of the treatment of several types of cancer, in
which an improvement in the quality of life of the patients has been reported (18,19). Additionally, several recent reports have demonstrated that OOS exhibits in vitro and in vivo antitumoral action in various tumor models, including HER2 positive breast cancer (13) and acute myeloid leukemia (20). These two tumor types are dissimilar at the molecular and cellular levels; however, the clear antitumoral action of OOS has been reported in the two. In addition, the antitumor action appeared to have been mediated by the slowing of cell cycle progression induced by an increase in expression of the cell cycle inhibitor p27. Furthermore, a recent clinical study revealed preliminary data that suggested that OOS may be useful in patients with end-stage HCC (18).

On the basis of these precedents, the present study aimed to explore the potential antitumoral effect of OOS in preclinical models of HCC. The present study demonstrated that OOS exhibits antitumoral effects in various HCC cellular models in vitro. In addition, when tested in vivo, an antitumoral effect was also demonstrated in terms of tumor growth reduction. Combinational studies with sorafenib, the standard of care for the treatment of HCC, indicated increased antitumoral activity in the combination, providing the rationale to additionally explore the clinical value of this therapy in HCC.

Materials and methods

Reagents and antibodies. The Dulbecco's modified Eagle's medium (DMEM), MTT, hematoxylin and eosin were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). The fetal bovine serum (FBS) and antibiotics were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and Immobilon P polyvinylidene fluoride membranes from Merck Millipore. OOS was provided by Catalysis, S.L. (Madrid, Spain). The other generic chemicals were purchased from Sigma-Aldrich, Roche Applied Science (Mannheim, Germany) or Merck Millipore.

The origins of the various antibodies used in the Western blot analyses are as follows: Anti-GAPDH (cat. no. sc-166574; 1:10,000) and anti-cyclin B (cat. no. sc-245; 1:5,000) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and the anti-phospho-histone H3 (cat. no. 06-570; 1:3,500) from Merck Millipore. The horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; cat. no. 170-6515; 1:20,000; and goat anti-mouse; cat. no. 170-6516; 1:10,000), were obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

Cell culture, cell proliferation measurement, protein purification and western blot analysis. The HepG2 and Huh7 HCC cell lines were donated by Dr Molin­nodo (University of Salamanca, Salamanca, Spain) and grown in DMEM supplemented with 10% FBS and antibiotics. The cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂,95% air.

The proliferation of HCC cells was examined using a modified MTT metabolism assay (13). The cells were plated in triplicate and treated the next day as indicated, with various concentrations of OOS alone or in combination with other drugs: Sorafenib (LC Laboratories, Woburn, MA, USA) 0.1, 0.3, 1 or 3 μM; docetaxel (Hospira UK Ltd., Leamington Spa, UK) 1 or 3 μM; and cisplatin (Pharmacia Grupo Pfizer, Madrid, Spain) 2 and 20 μM. On the day of the experiment, MTT was added to the wells at 0.5 mg/ml and incubated at 37°C for 1 h. The MTT-formazan crystals were dissolved in DMSO and the absorbance of the samples was recorded at 570 nm using a Tecan spectrophotometer with X-Fluor 4 (version 4.50; Tecan Trading AG, Männedorf, Switzerland) software. A minimum of 3 wells were analyzed for each condition, and the results are presented as the mean ± standard deviation (SD) of a representative experiment repeated at least twice. In order to prepare the cells for protein analyses, they were washed in PBS and lysed in an ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 11% Nonidet P-40, 20 mM Tris, pH 8.0, 1 mM pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate). Protein quantification, SDS-PAGE and western blot analyses were performed as previously described (21). For the western blot analysis, a total of 50 μg protein were probed with the indicated antibodies.

Transfection and fluorimetric quantification. The HepG2 and Huh7 cells were transfected with a plasmid coding for the luciferase gene (pCDNA3.1-Luc) using the jetPEI® reagent (Polyplus-transfection®, Illkirch, France) according to the protocol of the manufacturer. The plasmid coding for the luciferase gene (pCDNA3.1-Luc) was donated by Dr J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). Positive transfected cells were obtained subsequent to selection with the appropriate antibiotic, 450 μg/ml hygromycin. Once the clones had been isolated they were independently harvested by cloning rings, grown in multiwell plates and expanded. Hygromycin positive clones were tested for the presence of the luciferase gene by adding 150 μg/ml luciferin to the culture media, and then reading the luminescence in triplicate wells using a Synergy4 multi-mode microplate reader with Gen5 1.05 software (BioTek Instruments, Inc., Winooski, VT, USA). Each clone was analyzed at least twice and one of them, HepG2-Luc#22, was chosen for further experiments. In addition, a previously published MCF7-luciferase expressing clone was established as previously described (22) and analyzed in parallel as a positive control. The correct behavior of these clones in terms of general aspect, proliferation (measured as MTT metabolism) and cell cycle profile was determined before further proceeding.

Cell death, cell cycle and cell synchronization and release experiments. To analyze apoptotic cell death, the cells were treated for 48 h with the indicated treatments (OOS; dilutions, 1:25, 1:50 or 1:100 in culture media), resuspended in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH; 140 mM NaCl; 2.5 mM CaCl₂; pH=7.4) containing 5 μl Annexin V-fluorescein isothiocyanate (BD Biosciences) and 5 μl 50 μg/ml propidium iodide (PI), and stained at room temperature for 15 min. A total of 50,000 cells were acquired using a BD Accuri C6 flow cytometer and C6 software (version 1.0.264.21; BD Biosciences).

For cell cycle analysis by flow cytometry, the ethanol-fixed cells were stained with 5 μg/ml PI and 250 μg DNase-free RNase. A total of 50,000 cells were acquired as previously described. For the cell cycle synchronization experiments, the cells were arrested at various phases of the cell cycle as previously described (21). Thus, for G₂-M synchronization,
nocodazole treatment was used, whereas for G1-S arrest a double thymidine block was carried out. Subsequent to synchronization, the cells were washed twice in PBS and released in normal medium, or in medium supplemented with OOS (dilution, 1:25), and the cell samples were harvested after 1, 2, 3, 6, 9, 12 or 24 h for additional flow cytometry or western blot analysis.

In vivo experiments. For the animal studies, 12 7-week-old female athymic mice (BALB/c nu/nu) weighing between 18 and 20 g, were purchased from Charles River Laboratories (Wilmington, MA, USA) and kept in pathogen-free housing at Universidad de Salamanca Animal Care Facility (Salamanca, Spain). All animal experiments were performed according to the institutional guidelines and protocol approved by the Ethics Committee of Universidad de Salamanca (Salamanca, Spain). A total of 1 week subsequent to the purchase of the mice, they were injected with the appropriate cells to generate tumors. For the xenograft model, 1x10⁶ HepG2 cells resuspended in 50 µl DMEM and 50 µl Matrigel were subcutaneously injected into the right caudal flank of each mouse. When the tumors became palpable, the mice were randomly assigned into two groups (six per group), which received either vehicle alone, the control group, or 100 µl OOS per 20 g weight of mouse. The treatments were administered with a once daily schedule (Monday to Friday) by oral gavage until the humane endpoint (tumor volume, 2,000 mm³) was achieved by the control group and they were subsequently sacrificed by isoﬂurane euthanasia. The mice were weighed and the tumors were measured twice a week with a digital caliper (Proinsa, Vitoria, Spain). Tumor volumes were calculated using the formula: V= (L²/2) x (W/2)² x 4/3 x π, where V = volume (cubic mm), L= length (mm) and W= width (mm). Subsequent to the sacrifice of the mice, the tumor tissue was resected and immediately frozen at -80°C.

For the disseminated model, HepG2-Luc cells were directly injected into the liver of 14 BALB/C nu/nu mice who were anesthetized with ketamine and xylazine, and a 5-mm incision was carried out in the upper left ventral region of the abdomen, through the skin and the muscle layer to access the abdominal cavity. A total of 0.5x10⁶ HepG2-Luc cells resuspended in 25 µl DMEM and 25 µl Matrigel were injected in the liver, which was observed through the incision. The muscle and the skin layers were independently sutured, the region was cleaned and the mice were treated with buprenorphine, used as an analgesic. The correct localization of the cells was analyzed in vivo by bioluminescent imaging, performed with the IVIS 50 imaging system (Xenogen Corporation, Alameda, CA, USA), and the results were analyzed using Living Image software (version 4.1; Perking Elmer, Boston, MA, USA). The mice were intraperitoneally injected with 150 mg/kg aqueous solution of D-luciferin (Perking Elmer, Boston, MA, USA) and anesthetized by isoﬂurane inhalation, and images were captured. The status of the mice, including general status and weight, and tumor growth were determined twice a week.

Histological and immunohistochemical (IHC) analyses. Representative tumor areas were fixed in formalin, embedded in paraffin, cut into 2-3-µm sections and either stained with hematoxylin and eosin or prepared for IHC, which was performed as previously described (23). Thus, two cell conditioning periods of 8 min at 95°C and 4 min at 100°C on a hot plate using Tris-EDTA, pH=8, buffer were performed on previously dewaxed formalin-fixed paraffin-embedded tissue sections. Sections were incubated for 42 min at 37°C with a 1:50 dilution anti-Ki67 antibody (Master Diagnostica, Granada, Spain; cat. no. MAD-000310QD), and the staining was performed with the IHC 3,3'-diaminobenzidine system (Ventana Medical Systems, Tuscon, AZ, USA). The results were evaluated by pathologists blinded to the clinicopathological and molecular data. The extension of the necrotic area present in each tissue sample was assessed by measuring the total tumor and necrotic areas. The tumors were scanned and the percentage of the necrotic region was normalized to the total area using the dotSlide system and dotSlide 2.1 software (Olympus Corporation, Tokyo, Japan). These procedures were performed by independent personnel of the pathology unit of the University of Salamanca (Salamanca, Spain).

Statistical analysis. Each condition was analyzed in triplicate or quadruplicate and data are presented as the mean ± SD of ≥3 independent experiments. Comparisons of continuous variables between two groups were performed using a two-sided Student’s t test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of OOS on the proliferation of HCC cell lines. To study the potential effect of OOS on HCC, HepG2 and Huh7 HCC cell lines were treated for various days with increasing amounts of OOS, and the cellular response in terms of cellular proliferation was assessed. In those conditions, OOS reduced MTT metabolism, used as an indicator of cell proliferation, in the two cell lines (Fig. 1). The effect was time- and dose-dependent. The HepG2 cell line was more sensitive to the antiproliferative effect of OOS, compared with the Huh7 cell line. Treatment of HepG2 cells with a 1:100 dilution of OOS inhibited cell proliferation by 50% subsequent to three days of treatment (P=0.00082; Fig. 1A), whereas an equal dose of OOS did not substantially affect the proliferation of Huh7 cells (P=0.21; Fig. 1B). Due to the higher sensitivity of the HepG2 cell line to OOS, the present study preferentially used the aforementioned cell line as the principal model to explore the mechanism of action of OOS in HCC.

Effect of OOS in combination with other anti-HCC treatments. In the majority of the cases the success of antitumoral treatments is based on the combination of various agents. For that reason, the present study investigated if the antitumoral effect of OOS on the HepG2 cell line was increased by other drugs commonly used in the treatment of cancer. Since sorafenib is the recommended systemic treatment for patients diagnosed with advanced HCC, the present study tested the effect of OOS in combination with sorafenib. The simultaneous use of the agents was more efficient with respect to inhibiting the cell growth, compared with any of the individual treatments (Fig. 2A). OOS was also evaluated in combination with other drugs that had exhibited efficacy in the treatment of patients with HCC, including the antitumoral agents cisplatin (24) and...
However, treatment with OOS in combination with these agents did not substantially improve the effect of the individual treatments (Fig. 2B).

**Effect of OOS on apoptotic cell death and cell cycle progression.** A decrease in MTT metabolization may be caused by an increase in cell death, cell cycle arrest, or a combination of the two. To additionally investigate the mechanism of action of OOS in HCC cells, apoptotic cell death was initially assessed. The HepG2 cells were treated with increasing concentrations of OOS and apoptotic cell death was determined by double staining with Annexin V and PI. No major changes in the various populations were observed in any of the concentrations tested, indicating that there was no apoptotic cell death induced by OOS observed under the experimental conditions of the present study (Fig. 3A).

The current study then investigated if OOS induced cell cycle arrest in HCC cells. The cells treated for 48 h with increasing concentrations of OOS were fixed and the cell cycle profile was analyzed by PI staining of the DNA. A decrease in the number of cells in the G1 phase of the cell cycle was observed. In addition, treatment with OOS resulted in an increase in the number of cells present in the G1-M section of the histogram (Fig. 3B). These results indicated that the antitumoral action of OOS was due to an effect on cell cycle progression, without an apparent effect of OOS on cell viability.

To more precisely map the effect of OOS on cell cycle progression, synchronization and release experiments were carried out. Two types of synchronization procedures were used to arrest cells in various cell cycle phases. Firstly, HepG2 cells were synchronized in the G1 phase of the cell cycle by double thymidine treatment (21), and then released from the double thymidine block by incubation in normal medium, or in medium supplemented with OOS. A section of the sample was fixed to analyze cell cycle progression by flow cytometry, and the other section was lysed in order to explore the biochemical markers of each cell cycle phase using western blotting. Cells were observed to progress through the cell cycle more slowly subsequent to OOS treatment (Fig. 4A and B). For example, when the cell cycle distribution was analyzed using flow cytometry 9 h subsequent to releasing the block, the majority of the cells had completed the cell cycle in the control condition, as the majority of the cells were already in the G1 phase of the cell cycle, with 2N DNA content. At that time, the majority of the OOS-treated cells were in the G2-M phase, with 4N DNA content (Fig. 4A). This observation was confirmed when the expression levels of key cell cycle markers were determined by western blot analysis. Thus, at the aforementioned time point, the levels of the G2-M markers cyclin B and phospho-histone H3 were higher in the OOS-treated cells, compared with the control population, and demonstrated a larger number of cells in the G2-M phase (Fig. 4B).

The delay of the cell cycle progression induced by OOS was additionally confirmed by nocodazole synchronization experiments. This is a reversible microtubule polymerization inhibitor that arrests the cell cycle in the M phase, since cells are unable to form the mitotic spindle in the presence of nocodazole and the spindle checkpoint becomes active (27). When HepG2 cells were synchronized using nocodazole and released into OOS-containing media, a delay in mitotic exit was observed by flow cytometry and by western blot analysis of key mitotic markers (Fig. 4C and D). A total of 1 h subsequent to the release into the media, ~1/2 of the cells were in G1 in the control condition, whereas none of the cells had reached G1 in the OOS-treated group as observed by flow cytometry cell cycle analysis (Fig. 4C). In addition, a delay in the degradation of the G2-M markers cyclin B1 and phospho-histone H3 by western blot analysis was observed subsequent to OOS treatment, confirming that the cell cycle was slowed under that condition (Fig. 4D).

**Effect of OOS treatment on xenograft tumor models.** To determine if OOS exerted an effect on HCC growth in vivo, two distinct mouse models were developed. In the first one, HepG2 cells were engrafted in the flanks of BALB/c nu/nu athymic mice. Subsequent to three days, when the tumors became palpable, the mice were randomly assigned into two groups,
Figure 2. Effect of OOS in combination with other drugs in hepatocellular carcinoma. (A) OOS is more effective when used in combination with sorafenib. HepG2 cells were treated for 48 h with increasing concentrations of OOS, sorafenib or their combination, and the effect was determined by MTT uptake. (B) Similarly, the effect of OOS in combination with the antitumoral drugs CPT or TXT was determined. Mean absorbance values of untreated samples were taken as 100% and the mean values were then referred to that. Data are presented as the mean ± standard deviation of triplicates of an experiment that was repeated ≥3 times. CPT, cisplatin; TXT, docetaxel; OOS, Ocoxin® oral solution; soraf, sorafenib.

Figure 3. OOS does not induce apoptotic cell death or cell cycle arrest in vitro. (A) HepG2 cells were treated with the indicated concentrations of OOS and, subsequent to 48 h, were harvested and stained with Annexin V and PI to determine apoptotic cell death. (B) OOS does not induce major cell cycle arrest. Asynchronously growing HepG2 cells were treated for 48 h with the indicated dilution of OOS. Cells were then harvested, ethanol-fixed and their cell cycle status determined by PI staining. OOS, Ocoxin® oral solution; PI, propidium iodide.
one receiving daily oral treatment with 100 µl OOS/20 g body weight and the other, considered the control group, administered 100 µl water/20 g body weight. The tumors in the mice receiving OOS treatment grew at a slower rate, as compared with the tumors in the untreated control mice (Fig. 5A). Notably, and due to the aggressive characteristics of the HCC cells in vivo, all mice lost weight throughout the experiment. However, that effect was independent of OOS administration as there were no differences observed in the general behavior of the two groups (Fig. 5B).

To investigate the reason leading to the diminished tumor growth observed in OOS-treated mice, several tumors were resected and fixed subsequent to the sacrifice of the mice, and stained with the proliferation marker Ki-67. When the whole tumor section was observed, a difference in proliferative vs. necrotic regions was identified (Fig. 5C). Thus, when the total necrotic area was measured and normalized to total tumor area, an increase in necrosis was identified in the tumors from OOS-treated mice (Fig. 5D). In addition, OOS induced an evident decrease in the percentage of Ki-67 positive cells present in the proliferative region of the tumor; this was a statistically significant reduction compared with the control untreated tumors (P=0.049; Fig. 5E).

A second mouse model was used to follow the growth and dissemination properties of HepG2 cells in vivo. For this model, HepG2 cells were transfected with a plasmid coding for the luciferase gene, so that cells carrying the gene would emit light in the presence of luciferin. HepG2 and Huh7 cells positively expressing the luciferase gene, HepG2-Luc and Huh7-Luc, respectively, were isolated and luciferase expression was assessed. One of the clones expressing high levels of the transgene, HepG2-Luc#22 (Fig. 6A), was selected for additional investigation. This clone, although expressing the luciferase gene, exhibited normal proliferation and cell cycle status compared with the parental cells (data not presented).

HepG2-Luc cells were orthotopically placed in the liver of BALB/c nu/nu athymic mice by a small incision in the skin and the direct injection of HepG2 cells into the liver. The correct location of the cells was verified in vivo by bioluminescent imaging (Fig. 6B). The progression of the tumors was followed...
by a luciferin injection twice a week. Although the tumors were
intrahepatically injected, they rapidly disseminated throughout
the abdomen, as demonstrated by imaging (Fig. 6B) and symp-
toms including an enlarged abdomen due to the accumulation
of ascitic fluid (data not presented). In addition, an abdominal
inspection of the mice revealed the infiltration of the peritoneal
cavity by tumoral masses (Fig. 6C). In general, the liver was
normal (Fig. 6D, top image) and at the time of sacrifice only
1/14 of the mice that were analyzed exhibited a major hepatic
lesion (Fig. 6D, lower image, black arrow).

A total of five days subsequent to injection the mice were
randomly assigned into two groups that received water or OOS
(200 µl/20 g body weight) by daily oral gavage. The control
and the OOS-treated tumors were aggressive; however, the
OOS-treated tumors appeared to be less aggressive, as the
control mice required sacrifice prior to the OOS-treated mice
(Fig. 6E).

Discussion

Even subsequent to curative resection of HCC in early-stage
disease, ~70% of patients exhibit recurrence after five years (2),
increasing the requirement for the development of novel drugs
for use in an adjuvant setting. No effective neoadjuvant or
adjuvant treatment options that reduce the risk of recurrence
are currently available. Additionally, in previous years adop-
tive immunotherapy and retinoids have been used with some
success in HCC (28, 29), although the results have been diffi-
cult to reproduce.

The present study was conducted in order to explore
the potential antitumoral action of OOS in HCC, following
several precedents. Firstly, our previous studies demonstrated
the antitumoral value of OOS in highly proliferating cells,
including breast cancer and acute myeloid leukemia (13, 20).
Secondly, numerous reports have demonstrated the effects
of epigallocatechin-3-gallate on HCC models inducing cell
death (30), suppressing hepatocellular carcinoma growth or
dissemination. The present study, using two HCC cell lines,
observed that OOS induced a decrease in MTT metaboliza-
tion, indicative of reduced proliferation in the HCC-derived
cell lines tested. In addition, combinatory studies using OOS
and standard of care drugs used in the therapy of HCC indi-
cated that OOS potentiated the effect of sorafenib, one of the
principal drugs used to treat advanced-stage HCC.

Mechanistically, the decrease in MTT metabolization
caused by OOS appeared to be cell death-independent, since
no major apoptotic population was detected in those condi-
tions. This was a noteworthy result as one of the product
components is epigallocatechin-3-gallate, which has formerly been reported to induce apoptosis in HCC cells (30). The reason for this discrepancy remains unknown, but may be associated with the distinct experimental conditions used in these studies, including the doses of the products employed. While OOS did not induce a noticeable effect on apoptosis, it exhibited a clear effect on cell cycle progression and that is a possible mechanism underlying the antitumoral action of OOS (30). The effect of OOS on cell cycle progression was more evident when analyzing the action of OOS on synchronized cells. In cells synchronized in G1 by a double thymidine block protocol, OOS delayed G1-S progression. In addition, the nocodazole block experiments also indicated that OOS delayed the progression from the M to G0 phase. The observation that OOS affected progression along various phases of the cell cycle is notable, and suggests that OOS as a companion product may be used with other agents acting on more specific cell cycle phases. Therefore, the action of OOS in HCC appears to involve a delay in cell cycle progression rather than cell cycle arrest, which is in line with the conclusions observed when analyzing other tumor types (13,20).

The data presented in the current study indicates that OOS may be beneficial for patients with HCC, particularly in combination with standard of care drugs. This conclusion is supported by the in vitro data and by the observation that OOS also exerted an antitumoral action when administered to mice carrying xenografted HepG2 cells. The effect of OOS on this in vivo model resulted in a decrease in the rate of tumor growth, suggesting that OOS exerted a cytostatic effect, in line with the in vitro results on the mechanism of action of OOS. In addition, the larger areas of necrotic tumor tissue observed in the tissue samples from OOS-treated mice suggests that this compound may provoke damage to the tumoral tissue, either by acting directly on the tumoral cells or their supporting stroma. These findings are relevant as, together with recent data indicating that patients with terminal HCC exhibited a longer survival when administered OOS (18), it opens the possibility of using OOS to delay tumor progression when used in combination with standard of care therapies. In
addition, the preventive effect on HCC previously reported for epigallocatechin-3-gallate (8), raises the possibility of using OOS in patients with cirrhosis or patients previously affected with hepatitis B in order to combat the progression of these diseases into HCC. Future studies may assess this hypothesis using models of the aforementioned diseases at pre-tumoral stages.

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