Organic extract of *Geodia cydonium* induces cell cycle block in human mesothelioma cells

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Abstract. The serious side effects caused by chemotherapeutics and the development of cancer chemoresistance represent the most significant limitations in the treatment of cancer. Some alternative approaches have been developed in recent years, which are based on natural compounds, and have allowed important advances in cancer therapeutics. During the last 50 years, sponges have been considered a promising source of natural products from the marine environment, representing ~30% of all marine natural products. Among sponges, the Mediterranean species *Geodia cydonium* represents a potential source of these type of products with considerable biotechnological interest as pharmaceutical agents. The present study demonstrated the antiproliferative effect of an organic *G. cydonium* extract (GEOCYDO) against three human mesothelioma cell lines, MSTO-211H (MSTO), NCI-H2452 (NCI) and Ist-Mes2 (Mes2), which differ in their sensitivity (MSTO and NCI) and resistance (Mes2) to standard combined treatment with cisplatin and piroxicam. To this aim, the activity of the extract was evaluated by analyzing its effects on cell viability, cancer properties and cell cycle progression by means of colony formation assay, cell cycle analysis and protein expression analysis. The results revealed, in mesothelioma, this extract was able to reduce self-renewal, cell migration and it could induce cell cycle arrest in G_0/G_1 stage, thus blocking cell proliferation. In conclusion, to the best of our knowledge, the present results indicated for the first time that GEOCYDO can contain active compounds able to affect cell proliferation in mesothelioma, suggesting that it could be considered as a potential novel drug source for cancer treatment.

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Introduction

In recent decades, the search for active compounds from natural sources, mainly for pharmacological applications, has represented an important challenge (1). The increasing incidence of severe diseases, such as cancer, demand an urgent need to discover new drugs. Standard drugs have notable toxicity and their use is often associated with tumor resistance, thus the development of more effective therapies is required. In addition, natural anticancer compounds, unlike synthetic drugs, are able to inhibit tumor growth with minimal side effects (2). Particularly intriguing is the identification of molecules from the marine environment to be used in pharmaceuticals. Notably, oceans cover >70% of the earth surface and display higher biodiversity than the terrestrial environment; therefore, they have become an interesting source for the discovery of novel drugs. To date, oceans are still a largely unexplored environment, suggesting them as promising candidates for sources of biologically active natural compounds (3,4). Notably, soft-bodied sessile marine invertebrates, such as sponges, are able to produce several secondary metabolites for their survival in different habitats, which are used to counterattack predators, and in competition for space and nutrients. These bioactive compounds could be useful in pharmacological, nutraceutical and cosmeceutical applications (5,6).

Sponges represent the most studied marine organisms as sources of bioactive compounds (1,3,4). Previous studies have reported the bioactivity of different marine sponge extracts in several diseases and some extracts have been used to produce commercial anticancer drugs (7). Among sponges, the bioactivity of the Mediterranean sponge *Geodia cydonium* has been poorly characterized. Our previous study reported the anti-inflammatory and anticancer effects of *G. cydonium* organic extracts on breast cancer cells (8,9).

To the best of our knowledge, the present study was the first to evaluate the antiproliferative potential of *G. cydonium* extract (GEOCYDO) in mesothelioma, which is a rare and aggressive type of cancer associated with exposure to asbestos fibers that exhibits high chemoresistance (10). The present study used three human mesothelioma cell lines, MSTO-211H (MSTO), NCI-H2452 (NCI) and Ist-Mes2 (Mes2), which differ in their sensitivity (MSTO and NCI) and resistance (Mes2) to standard combined treatment with cisplatin and piroxicam (11). The aim of the present study was to analyze the effect of GEOCYDO on mesothelioma, a type of cancer characterized by high chemoresistance. The present study indicated that the extract affects mesothelioma cell viability and that the fraction C could be the one responsible for its antiproliferative effects, being the most active against the three mesothelioma cell lines. Furthermore, preliminary chemical analysis of fraction C (12) revealed a complex metabolic profile, which requires further fractionation for identification of the active metabolite. To the best of our knowledge, the present study provided novel findings, as despite the large number of marine compounds assessed as drug candidates in various types of cancer (13), no previous study has referred to their use in mesothelioma.

Materials and methods

Collection of biological material. The present study did not involve protected species. *G. cydonium* (order, Tetractinellida; family, Geodiidae) samples were collected at 20 m in depth by scuba diving at the 'Parco Sommerso di Baia' (Naples, Italy). As soon as the samples were collected, they were stored at -20˚C until further analysis.

G. cydonium extraction. Lyophilized sponge tissue (200 g wet weight) was extracted with methanol at room temperature. After sonication (5 min, 59 KHz, 26˚C), the organic extract was dried under nitrogen flow and maintained at -20˚C until further use. The extraction step was repeated three times. The extract was filtered through Whatman filter paper to recover solvent residues, and was then evaporated at low pressure in a rotavapor at 28˚C and dissolved in methanol. The final extract was dried and stored at -20˚C until use. For the NMR analysis (Pulprog: zg), the dry extract was dissolved in deuterated methanol (CD_3OD) and transferred to a NMR tube. NMR spectra were recorded on Bruker DRX 600 spectrometer equipped with an inverse TCI CryoProbe (Bruker Corporation). Chemical shift values are reported in ppm (δ) and referenced to internal signals of residual protons (CD_3OD, ^1H 3.34).

Cell culture and chemicals. Human mesothelioma cell lines MSTO and NCI, and the human mesothelial cell line MeT-5A were grown in RPMI supplemented with 10% FBS (both from Euroclone SpA), glutamine (2 mM), sodium pyruvate and antibiotics (0.02 IU/ml penicillin and 0.02 mg/ml streptomycin). Human mesothelioma Mes2 cells were cultured in DMEM (Euroclone SpA), supplemented with 10% FBS, glutamine (2 mM), 1% nonessential amino acids and antibiotics (0.02 IU/ml penicillin and 0.02 mg/ml streptomycin). MSTO, NCI and MeT-5A cells were obtained from the American Type Culture Collection, and Mes2 cells were obtained from the Istituto Nazionale per la Ricercasul Cancro-Genova.

Fractionation of the methanolic extract of G. cydonium. A small amount of the active methanol extract (~40 mg) was subject to SPE using CHROMABOND® HRX cartridges (6 ml/500 mg; Macherey-Nagel) on a GX-271 ASPEC (Gilson, Inc.) (12). The extract was suspended in 1 ml distilled water and sonicated (59 KHz, 26˚C), for a few seconds in an ultrasonic bath before loading onto the column, which was previously conditioned with 3 ml methanol and equilibrated with 6 ml distilled water. This fractionation yielded five fractions (A, B, C, D and E) obtained by stepwise elution with H_2O (6 ml), CH_3OH/H_2O 7:3 (9 ml), CH_3CN/H_2O 7:3 (9 ml), CH_3CN (9 ml) and CH_2Cl_2/CH_3OH 9:1 (9 ml), respectively. The total extract (TE) and SPE fractions B-E were tested for cytotoxicity. The organic extract and fractions were then analyzed by thin layer chromatography (TLC) stained with $Ce(SO_4)_2$ and a preliminary chemical characterization was carried out by ^1H-NMR spectrum in CD_3OD. Each SPE fraction was dissolved in deuterated solvent (CD_3OD for GCYD-2B and 2C; $CDCl_3$ for GCYD-2D and 2E) and transferred to an NMR tube to acquire ^1H-NMR spectra (Pulprog: zg), as already reported for *G. cydonium* extract. Bidimensional NMR experiments heteronuclear single quantum coherence edited (HSQCed) (Fig. S1) and heteronuclear multiple bond correlation (HMBC) (Fig. S2) in CD_3OD were also acquired on the active SPE fraction C. NMR spectra were recorded on a Bruker DRX 600 spectrometer equipped with an inverse TCI CryoProbe. Chemical shift values are reported in ppm (δ) and referenced to internal signals of residual protons (CD_3OD, ^1H 3.34; $CDCl_3$, ^1H 7.26).

The extract and fractions were dissolved in 100 mM DMSO and dilutions were made to obtain the different concentrations to be tested, with a final concentration of 0.05% DMSO. Details on fractionation and analysis are reported in Figs. S1 and S2.

To evaluate the bioactivity of SPE fractions, MSTO, NCI and Mes2 cells were treated with 200 μg/ml of the four enriched samples (B-E) for 24 h at 37˚C; this concentration was selected as this concentration of the total extract did not affect cell viability.

Cell viability assay. For each cell line, ~$1x10^4$ cells/well were plated in 48-well plates and were treated with GEOCYDO at different concentrations (50, 150, 300 and 500 μg/ml) for 16, 24 or 48 h at 37˚C in humified atmosphere containing 5% CO_2. For fraction bioactivity analysis, cells were treated with 200 μg/ml SPE fractions B, C, D and E for 24 h at 37˚C. Subsequent analysis with 50, 100, 150 and 200 μg/ml fraction C for 24 h at 37˚C was performed done to establish the half maximal inhibitory concentration (IC_{50}) concentration. For all experiments, cells treated with the same amount of

vehicle (0.1% DMSO; MilliporeSigma) present in the extract were used as a control.

Cell viability was evaluated counting live cells using MTS assay (CellTiter 96; Promega Corporation) according to the manufacturers' instructions. For the MTS assay, treated cells were incubated with 20 μl MTS reagent for 2 h at 37˚C. The absorbance was recorded on a microplate reader at a wavelength of 490 nm (VICTOR Multilabel Plate Reader; PerkinElmer, Inc.). All experiments were performed in triplicate and data are expressed as the mean ± SD.

Colony formation assay. Colony formation was assessed as previously reported by our group (14). For each cell line, 500 cells/well were plated in six-well plates, incubated for 7 days and then treated with 500 μg/ml GEOCYDO for 24 or 48 h under culture conditions before replacing the media. The growth was assessed for a further 7 days and then crystal violet was used to stain the colonies, which were successively counted. Briefly, cells were fixed with formaldehyde (3.7%) for 10 min at room temperature, then washed with PBS and stained for 10 min with crystal violet (0.5%) at room temperature. The absorbance was measured at 595 nm using a microplate reader (Cytation3 ASHI; BioTek Corporation). A scanner (Epson Stylus Photo, PX 650; Epson) was used to capture images of representative plates. All experiments were performed in triplicate and data are expressed as the mean ± SD.

Wound-healing assay. The wound-healing assay was performed as previously reported (14,15). For each cell line, ~$3x10^5$ cells/well were seeded in six-well plates. After overnight incubation at 37˚C, cells were at 90% confluence and wounds were created using a 200-μl pipette tip. After wound generation, cells were treated using the aforementioned culture medium, with 500 μg/ml GEOCYDO or 0.1% DMSO for 24 or 48 h. To analyze cell migration, at least six representative images for each scratch were taken in different areas at different time points. A phase contrast light microscope (DMI8; Leica Microsystems GmbH) was used to capture images of the representative plates. ImageJ software (version 1.52; National Institutes of Health) and its wound healing assay macro was used to measure wound healing. All experiments were performed in triplicate and data are expressed as the mean ± SD.

Cell cycle analysis. After overnight incubation, ~$7.5x10^5$ cells/well were plated in 100-mm plates, serum starved for 24 h and then treated with 500 μg/ml GEOCYDO for 16, 24 or 48 h at 37˚C. PBS was used to wash the cells, which were then fixed in cold 70% ethanol for 30 min at 4˚C. After centrifugation at 850 x g for 8 min at 4˚C, cells were washed twice with cold PBS and cell pellets were dissolved in 500 μl cold PBS. To ensure only DNA was stained, cells were digested for 30 min at 37˚C with 100 μg/ml RNase A. Propidium iodide (50 μg/ml) was then used to stain cells for 30 min at room temperature and the cells were then analyzed by flow cytometry (FACSCanto; BD Biosciences) using FACSDiva software (version 6.1.3; BD Biosciences). A total of $20x10^4$ events were recorded for each sample and the percentage of cell fractions in all cell cycle phases was calculating. All experiments were performed in triplicate and data are expressed as the mean ± SD.

Western blot analysis. Protein extracts were obtained from cells treated with GEOCYDO (500 μg/ml) for 16, 24 or 48 h at 37˚C as previously described (11). Total cell lysates (20 μg) were separated on 4-15% Tris-glycine gels by SDS-PAGE (Bio-Rad Laboratories, Inc.) at 100 V and proteins were then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked in 5% milk in TBS/5% Tween at room temperature for 1 h and were then probed overnight at 4˚C with the specific primary antibodies, and then with horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. no. A6154; MilliporeSigma) for 1 h at room temperature according to the manufacturer's indications. The primary antibodies used for western blotting include: Anti-cyclin E (cat. no. sc-481; Santa Cruz Biotechnology, Inc), anti-cyclin A (cat. no. sc-596; Santa Cruz Biotechnology, Inc.), anti-cyclin B1 (cat. no. sc-245; Santa Cruz Biotechnology, Inc.), anti-p21 (cat. no. 2947; Cell Signaling Technology, Inc.), anti-p27 (cat. no. sc-528; Santa Cruz Biotechnology, Inc.) and anti-β-actin (cat. no. 3700; Cell Signaling Technology, Inc.), which was used as a loading control, at the concentrations suggested by manufacturers (1:1,000). Clarity western ECL (Bio-Rad Laboratories, Inc.) was used to detect protein bands and the blots were semi-quantified with ImageJ software. All experiments were performed in triplicate and data are expressed as the mean ± SD.

Statistical analysis. Graph Pad Prism 6.0 (GraphPad Software, Inc.) analysis was used to evaluate the difference between control and treatment groups. One-way ANOVA was used to evaluate the significance of the differences among means. Dunnett's multiple comparison test with Bonferroni post hoc correction was used to assess the significance between each treatment group and the control group. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

GEOCYDO affects cell viability. To analyze the bioactivity of GEOCYDO on mesothelioma cells, the present study first determined, in a dose-response curve at 24 h, the amount of extract that had a lethal effect on MSTO cells. Results revealed that GEOCYDO had a IC_{50} of ~500 μg/ml in MSTO cells (Fig. 1A). Therefore, a concentration of 500 μg/ml was used for subsequent experiments. By contrast, the same concentration of GEOCYDO had no effect on wild-type Met-5A mesothelial cells (Fig. 1B). The present study also analyzed if the effects of GEOCYDO were increased over the time. As shown in Fig. 1C, cell viability decreased from 16 to 48 h; it was reduced by 75% in MSTO, 70% in NCI and 80% in Mes2 cells at 48 h compared with in the vehicle-treated cells.

Subsequently, cell proliferation and migration were analyzed to evaluate the anticancer potential of GEOCYDO. The results clearly showed that GEOCYDO was able to impair cell proliferation, reducing cellular self-renewal ability and long-term proliferative potential in all of the cell lines tested. Notably, treatment with 500 μg/ml GEOCYDO inhibited colony formation after 24 and 48 h, and the ability to produce colonies was reduced by ~50% after 24 h, and by 65, 60 and 70% after 48 h in MSTO, NCI and Mes2 cells, respectively (Fig. 2A and B). In addition, to measure the migratory

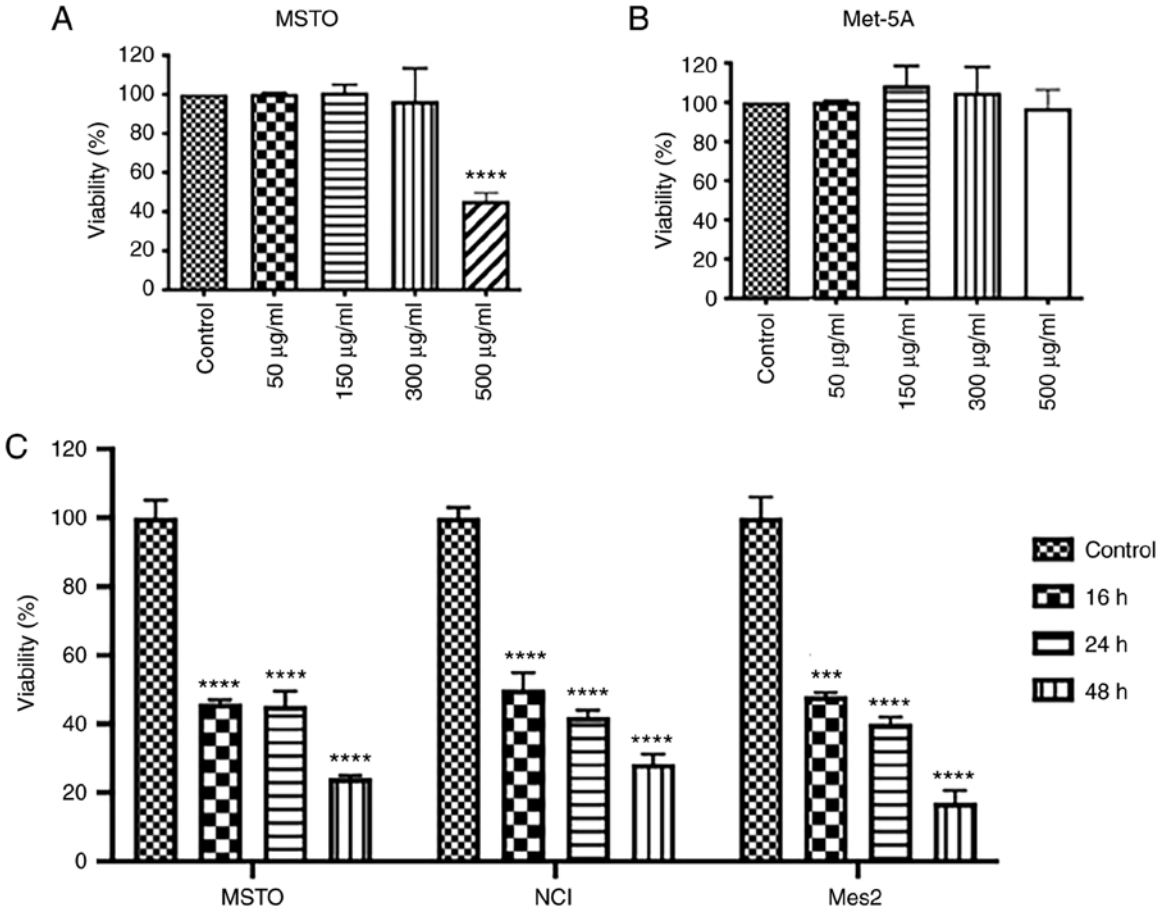

Figure 1. GEOCYDO treatment affects cell viability. Cell viability after treatment with various concentrations of GEOCYDO for 24 h in (A) MSTO or (B) Met-5A cells. (C) Cell viability after treatment of MSTO, NCI and Mes2 cells with 500 μg/ml GEOCYDO for different durations. Cells treated with vehicle only (0.1% DMSO) were used as a control. Data are presented as the mean ± standard deviation (n=3). ***P<0.005, ****P<0.001 vs. control. MSTO, MSTO-211H; NCI, NCI-H2452; Mes2, Ist-Mes2; GEOCYDO, *Geodia cydonium* extract.

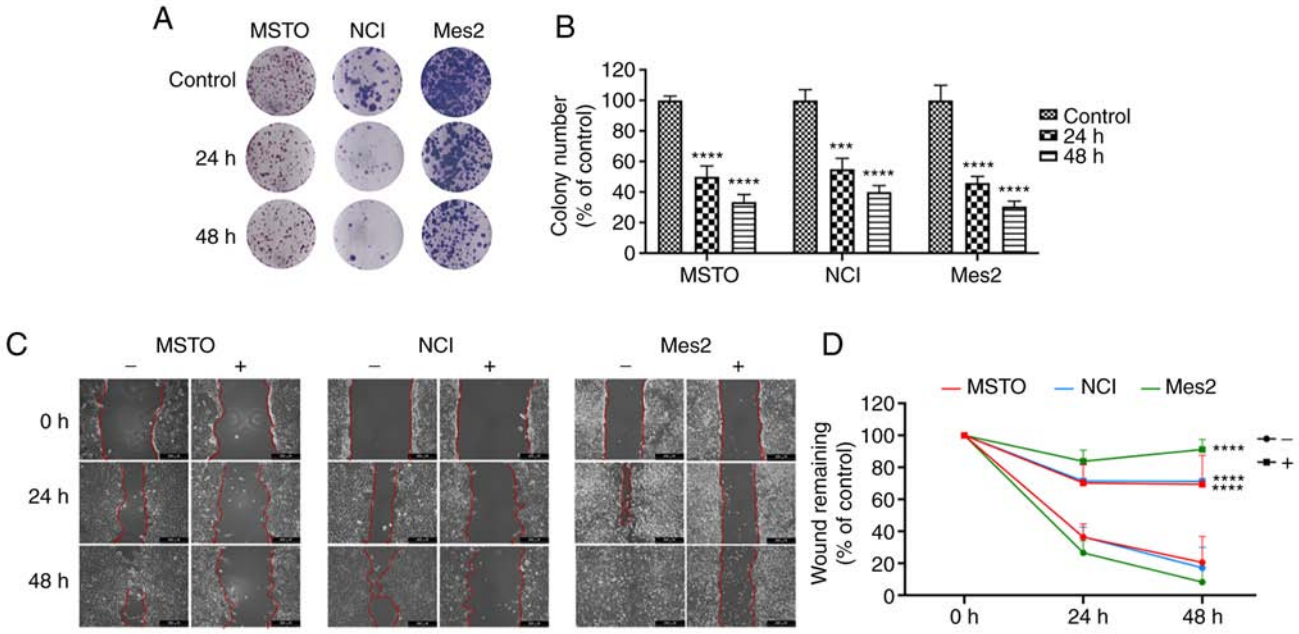

Figure 2. GEOCYDO treatment affects tumorigenic properties. (A) Colony formation assay on mesothelioma cell lines following GEOCYDO treatment. Representative plate images after crystal violet staining are shown. (B) Histograms of the average colony numbers. (C) Wound-healing assay. The wound closure rate was measured by detecting the closure distance at the time indicated in MSTO, NCI and Mes2 cells treated with 500 μg/ml GEOCYDO (+). Cells treated with vehicle only (0.1% DMSO) were used as a control (-). Representative micrographs under a phase contrast microscope are shown. Scale bar, 200 μm. (D) Quantification of wound gap distance. Data are presented as the mean ± standard deviation (n=3). ***P<0.005, ****P<0.001 vs. control. MSTO, MSTO-211H; NCI, NCI-H2452; Mes2, Ist-Mes2; GEOCYDO, *Geodia cydonium* extract.

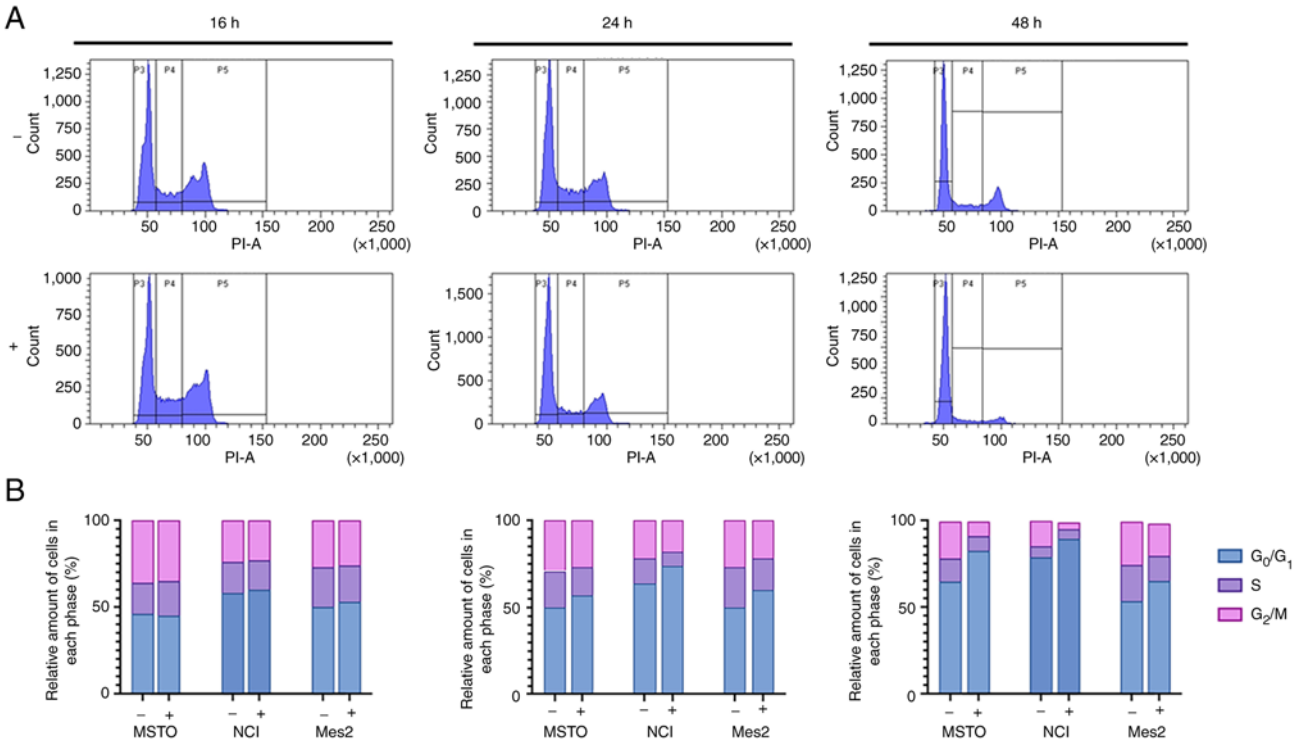

Figure 3. Effect of GEOCYDO on cell cycle progression. (A) Distribution of MSTO cell population during the cell cycle after 16, 24 and 48 h of treatment with 500 μg/ml GEOCYDO (+) analyzed by flow cytometry. Cells treated with vehicle only (0.1% DMSO) were used as a control (-). (B) Histograms of the cell cycle distribution in MSTO, NCI and Mes2 cells. Data are presented as the mean ± standard deviation (n=3). MSTO, MSTO-211H; NCI, NCI-H2452; Mes2, Ist-Mes2; GEOCYDO, *Geodia cydonium* extract; P3, G_0/G_1 cell population; P4, S cell population; P5, G_2/M cell population.

capability of cells, a wound-healing assay was performed, where scratched cells were treated with GEOCYDO. The results showed that GEOCYDO significantly inhibited the ability of cells to close the gap in all of the cell lines (Fig. 2C and D). By contrast, in the untreated cells, the wound gap was closed at the end of the treatment. Notably, this experiment does not completely distinguish if GEOCYDO treatment affects cell migration or proliferation, since it was not performed in low serum conditions; thus, proliferation inhibition was analyzed in more detail.

GEOCYDO extract induces G_0/G_1 cell cycle arrest. To explore the mechanisms underlying the inhibition of mesothelioma cells induced by GEOCYDO, cell cycle distribution was analyzed by flow cytometry. Cells were analyzed following treatment with GEOCYDO for 16, 24 or 48 h, in order to analyze cell proliferation modifications. As shown in Figs. 3 and S1, untreated MSTO cells exhibited a typical cell cycle distribution over time, whereas GEOCYDO treatment induced a cell cycle arrest at 24 and at 48 h, as shown by an increased percentage of the cell population in the G_0/G_1 phase. Notably, alongside the increase in the G_0/G_1 cell population, there was a corresponding reduction in the population of cells in S and G_2/M phases. Similar results were obtained with NCI and Mes2 cells (Fig. 3 and Fig. S3). Specifically, the percentage of cells arrested in G_0/G_1 phase was increased after 24 and 48 h in all of the cell lines analyzed; conversely, a slight decrease in the percentage of cells in S and G_2/M phases was found.

To assess the effects of GEOCYDO, the expression levels of different cyclins and of two CDK inhibitors (CDKIs), p21 and p27, which are crucial for cell cycle progression, were detected. In particular, the expression levels of cyclin E, which is required for cell cycle G_1/S transition, of cyclin A, which is needed for the G_2/M transition, and of cyclin B1, which is the mitotic cyclin, were detected. The analysis indicated a decrease in the expression levels of cyclin E in MSTO, NCI and Mes2 cells, which was in agreement with the observed cell cycle arrest (Fig. 4). Considering that cyclin E can regulate the passage between phase G_1 and S, these results confirmed that GEOCYDO blocked the transition between those two cell cycle phases. Cyclin A exhibited a similar decreased expression in MSTO and NCI cells, whereas it was not modulated in Mes2 treated cells compared with control cells. By contrast, cyclin B1 expression was decreased at 16 and 24 h in MSTO and NCI treated cells compared with in the control cells, and at all timepoints in Mes2 treated cells compared with in the control cells. The differences in the expression of cyclin B1 between the different cell lines could be related to the tumorigenicity of the cell lines. Finally, the decreased expression level of cyclins was accompanied by an increase in the expression levels of p21 and p27 in MSTO and NCI treated cells compared with in the control cells, and of p27 in Mes2 treated cells compared with in the control cells.

Analysis of GEOCYDO and SPE fractions. Spectroscopic analysis of GEOCYDO indicated that the active methanolic extract was very rich in metabolites. Proton signals in the region at low field showed the presence of aromatic compounds (blue arrows in Fig. 5), whereas the abundance of methyl singlets in the region between 2.7 and 3.7 ppm suggested the

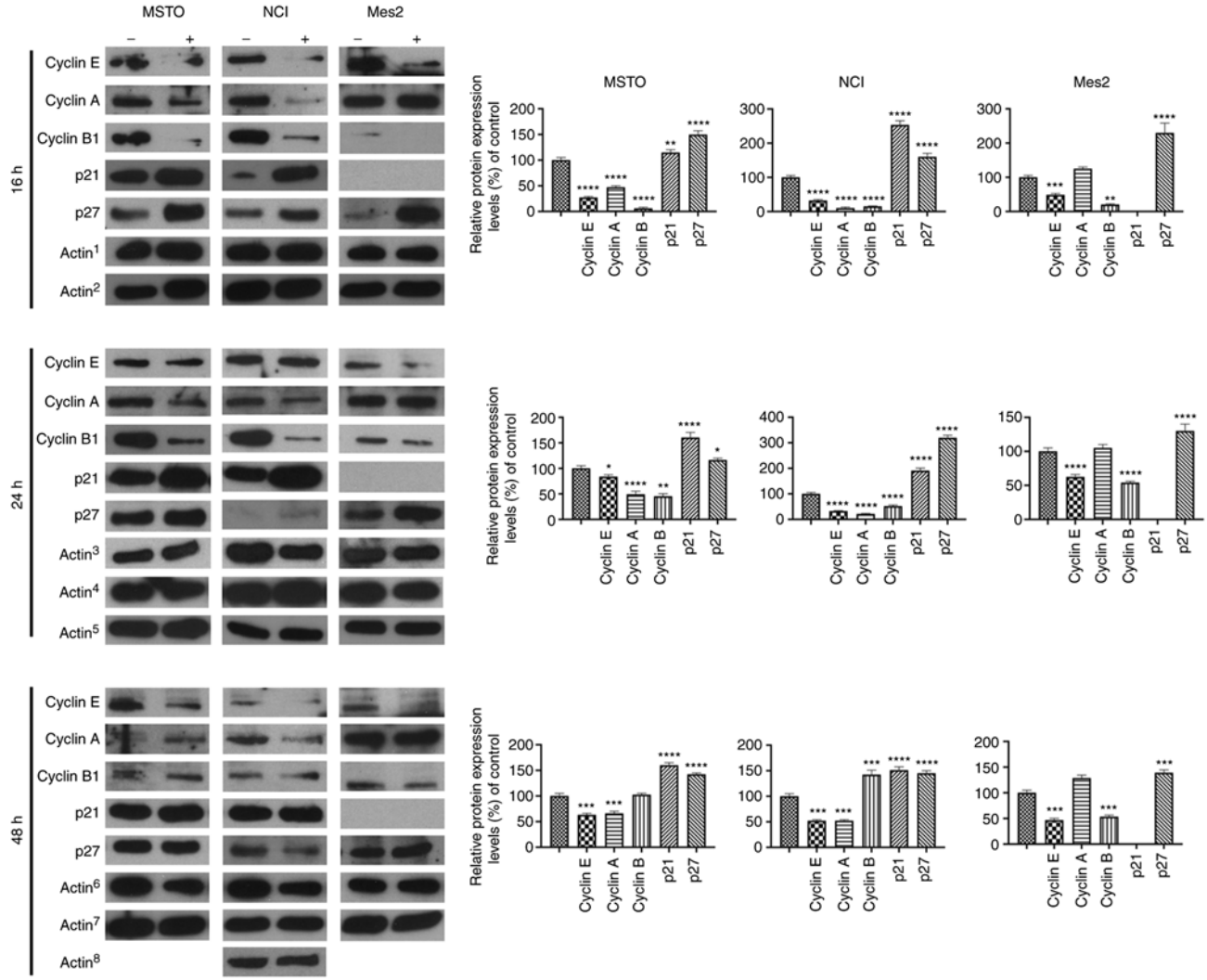

Figure 4. GEOCYDO induces cell cycle arrest at G_0/G_1 phase through modulation of cyclins and CDK inhibitors. Western blot analysis of the expression levels of cyclin E, cyclin A, cyclin B1, p21 and p27 16 h, 24 or 48 h after treatment with GEOCYDO. β-actin expression was used as a loading control. Actin[1] refers to the control for cyclin E, cyclin B1 and p21; Actin[2] refers to the control for cyclin A and p27; Actin[3] refers to the control for cyclin E, cyclin B1 and cyclin A (for NCI cells); Actin[4] refers to the control for p21; Actin[5] refers to the control for p27 and cyclin A (for MSTO and Mes2 cells); Actin[6] refers to the control for cyclin E, cyclin B1 and p21; Actin[7] refers to the control for p27 and cyclin A (for MSTO and Mes2 cells); Actin[8] refers to the control for cyclin A (for NCI cells). Histograms represent the relative expression levels relative to the control. All the controls were set at 100%. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs. control. MSTO, MSTO-211H; NCI, NCI-H2452; Mes2, Ist-Mes2; GEOCYDO, *Geodia cydonium* extract.

occurrence of heteroatom-containing compounds (purple arrows in Fig. 5). Fractionation of GEOCYDO by SPE-HRX column led to five new samples: Salts were eluted in fraction A, whereas the enriched fractions B-E, contained different classes of metabolites. As shown in Fig. 6, according to the expected resolution of the method (12), chemical analysis of the SPE fractions clearly indicated the presence of water-soluble metabolites in fraction A (mainly sugars); nucleosides and nitrogen-containing compounds in fraction B; complex lipids, including sphingolipids, lipopeptides, glycolipids and phospholipids in fraction C; sterols, terpenes, alcohols and fatty acids in fraction D; and triglycerides and neutral lipids in fraction E.

To evaluate the bioactivity of SPE fractions, MSTO, NCI and Mes2 cells were treated with 200 μg/ml of the four enriched samples (B-E) for 24 h; this concentration was selected as this concentration of the total extract did not affect cell viability. Cell viability analysis indicated that only fraction C was able to decrease viability in all cell lines (Fig. 7A). Subsequent analysis using various concentrations of fraction C (50-200 μg/ml) for 24 h confirmed its strong cytotoxicity on all mesothelioma cell lines analyzed, lowering the IC_{50} of GEOCYDO to ~150 μg/ml (Fig. 7B).

Preliminary ^{1}H-NMR and TLC analysis of the enriched fraction C indicated a prevalence in this fraction of polar minor metabolites (Fig. 6). However, although the composition of fraction C is very complex and produced a crowded NMR spectra with several overlapping signals, these data are consistent with the presence of molecules with cyclodepsipeptide or a macrolide skeleton (Figs. S2 and S3). In detail, spectroscopic data of this fraction revealed a chemical signature with several methine groups between 4 and 5 ppm, coupled with carbon between 50 and 60 ppm that could be diagnostic of amino acid skeletons, and various methine and methylene signals between 3.5 and 4.10 ppm coupled with oxygen-bearing carbon between 62 and 75 ppm (Fig. S1). In the HMBC spectrum (Fig. S2),

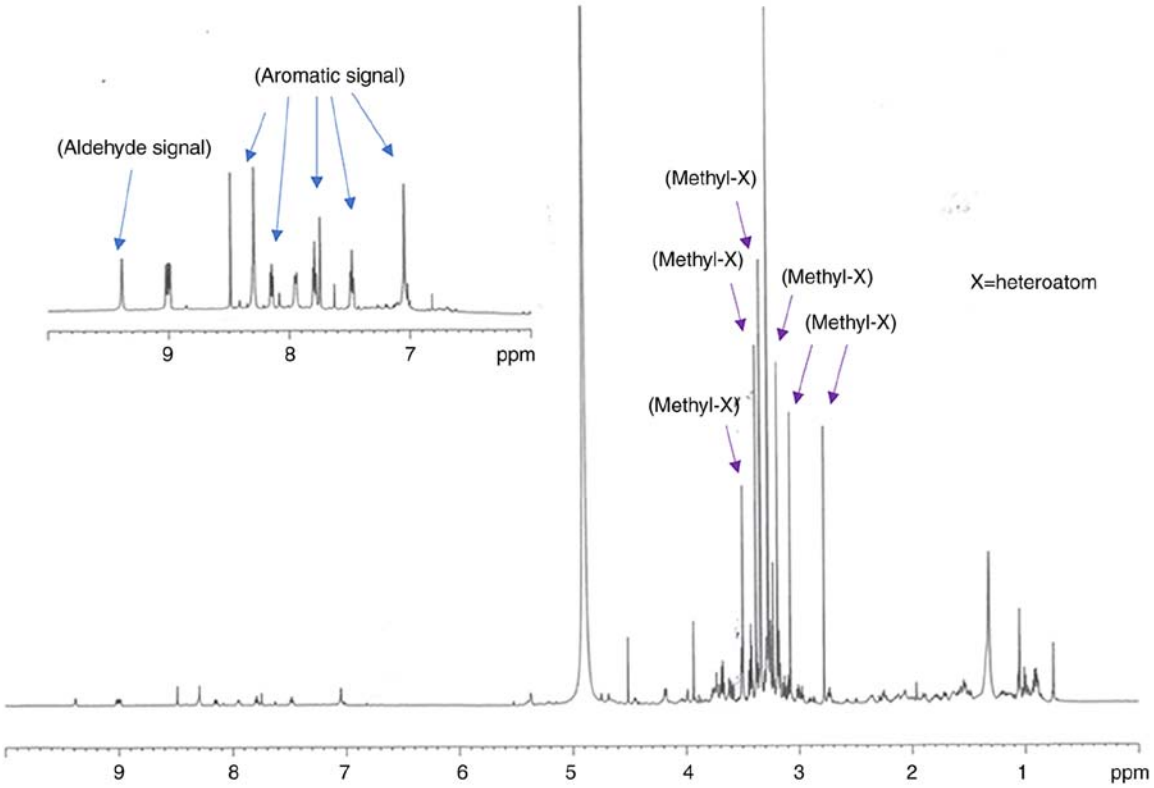

Figure 5. Chemical analysis of GEOCYDO. Proton nuclear magnetic resonance profile of methanolic GEOCYDO (total extract) in CD_3OD at Bruker 600 MHz. The spectrum mainly showed the presence of aromatic compounds (blue arrows), whereas the occurrence of heteroatom-containing compounds was suggested by methyl singlets (purple arrows) in the region between 2.7 and 3.7 ppm. X, heteroatom; GEOCYDO, *Geodia cydonium* extract.

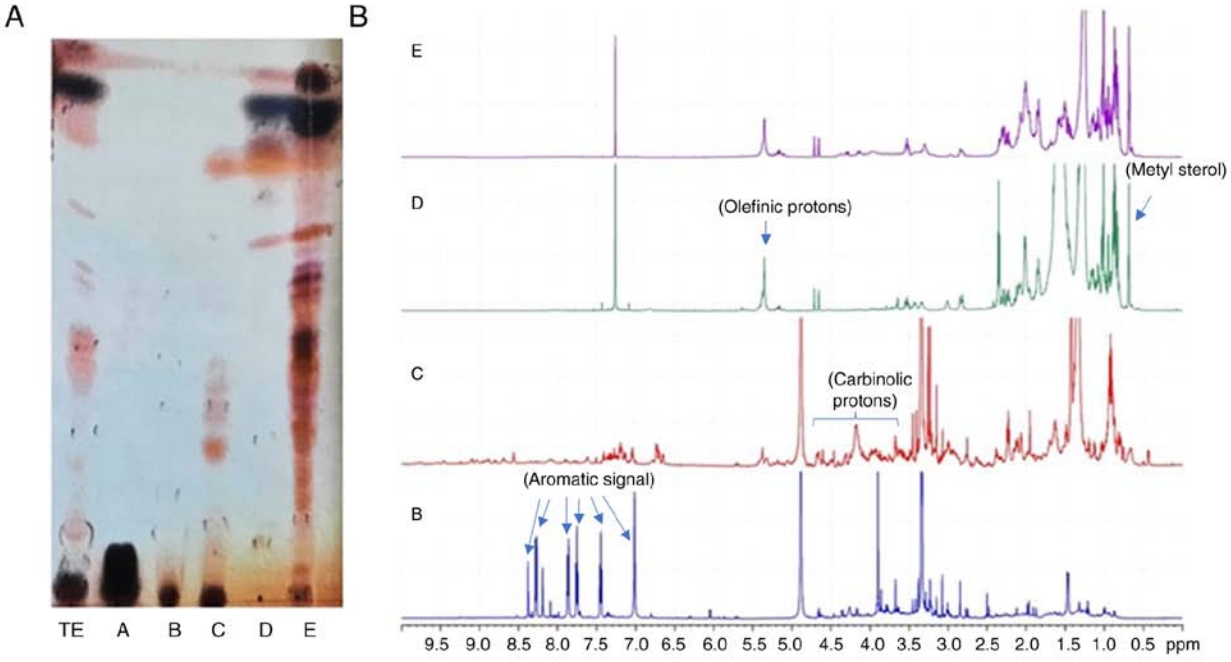

Figure 6. Chemical analysis of GEOCYDO fractions. (A) Thin layer chromatography in $CHCl_3$/MeOH/H_2O 65:25:4 of GEOCYDO TE and enriched SPE-HRX fractions (fractions A-E). (B) The metabolic profile acquired by proton nuclear magnetic resonance of the corresponding SPE-HRX fractions B-E (600 MHz, GCYD-2B and 2C were acquired in CD_3OD; GCYD-2D and 2E were acquired in $CDCl_3$). Blue arrows indicate the highest peaks. The blue bracket indicates carbinolic protons. GEOCYDO, *Geodia cydonium* extract; TE, total extract; SPE, solid-phase extraction.

these signals also showed correlations with ester functions at 170-175 ppm. In addition to methoxy moieties, the NMR spectra supported the presence of a carbonyl group below 210 ppm (Fig. S2), several methyl groups (^{1}H NMR signals at 0.57, 0.76, 0.82, 1.03 ppm; ^{13}C NMR signals at 25.3, 21.1, 15.9, 19.8 ppm), and various down-shifted protons between 6.5 and 7.3 ppm of

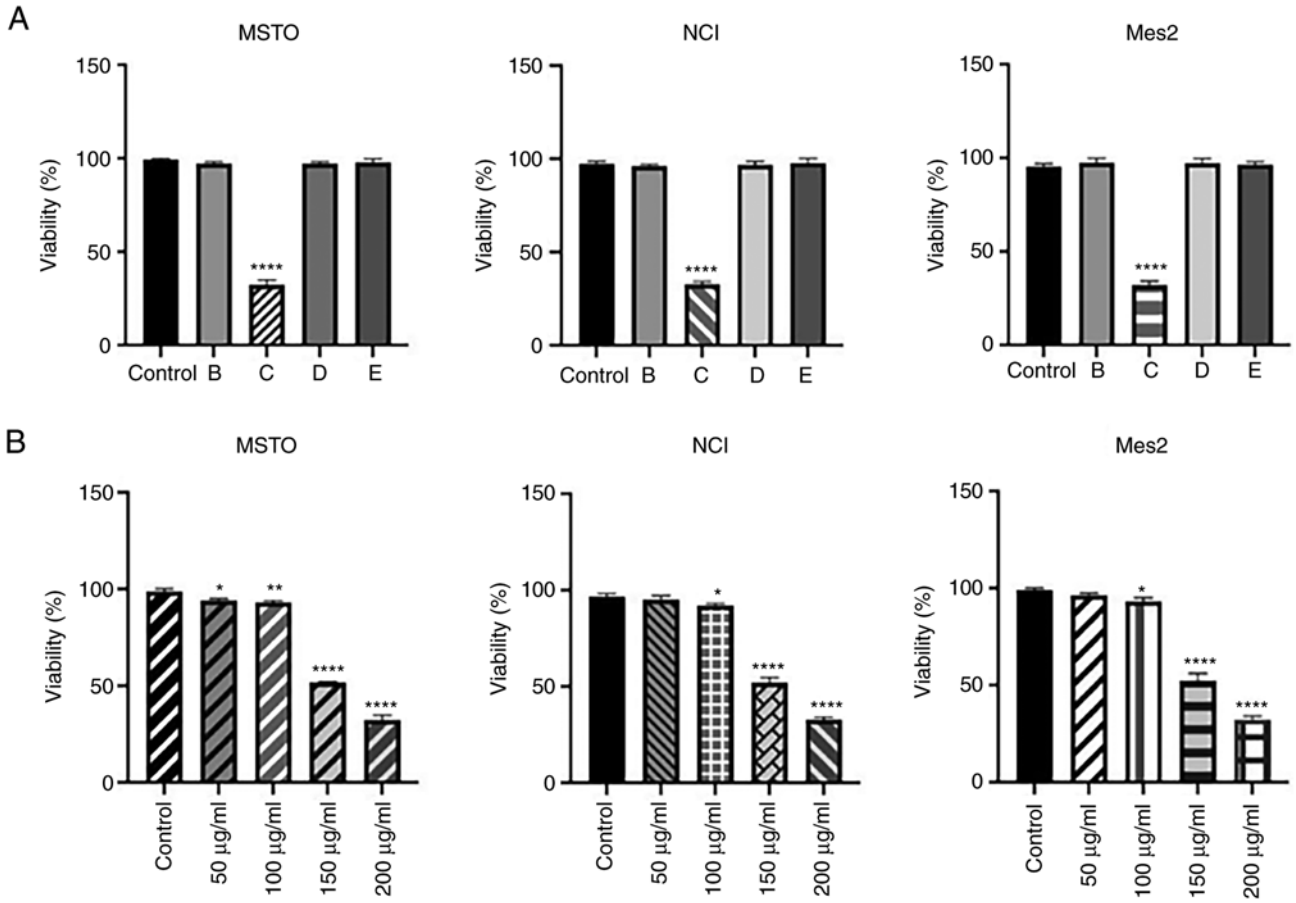

Figure 7. Fraction C affects cell viability. (A) MSTO, NCI and Mes2 cells were treated with 200 mg/ml solid-phase extraction fractions B-E for 24 h. (B) MSTO, NCI and Mes2 cells were treated with 50-200 μg/ml fraction C for 24 h. Cells treated with vehicle only (0.1% DMSO) were used as a control. Data are presented as the mean ± SD of independent experiments (n=3). *P<0.05, **P<0.01, ****P<0.0001 vs. control. MSTO, MSTO-211H; NCI, NCI-H2452; Mes2, Ist-Mes2.

conjugated unsaturated systems (Fig. S1). Unfortunately, the amount of fraction C available was not enough to complete the characterization of the active metabolite.

Discussion

To the best of our knowledge, the present study was the first to describe the *in vitro* antiproliferative effect of GEOCYDO extract on mesothelioma, a rare but very aggressive cancer characterized by high chemoresistance (10,14). Mesothelioma displays a long latency period (30-40 years) and a generally unfavorable outcome (10). Despite several reports in the literature on marine sponges (2,16-18), few studies have assessed the biotechnological applications of one of the major sources of bioactive natural products, *G. cydonium*. Our group previously reported that a methanolic extract from this sponge had an anti-inflammatory and pro-apoptotic effect on breast cancer cell lines (8,9).

The results of the present study clearly indicated that GEOCYDO was able to affect mesothelioma cancer properties acting on cell proliferation in a time-dependent manner. Cytofluorimetric analysis revealed that GEOCYDO induced cell cycle arrest at G_0/G_1 phase, which may induce inhibition of proliferation in mesothelioma. The present findings were confirmed by expression analysis of proteins involved in cell cycle arrest. Notably, specific alterations in the expression levels of cyclins A, E and B1, and of CDKIs p21 and p27, were detected (19).

Cell cycle progression is a finely tuned event regulated by protein kinase complexes containing cyclins and CDKs (20). In response to DNA damage, cell proliferation undergoes arrest until DNA is repaired and correctly replicated. Cell cycle arrest can occur at two specific checkpoints of the cell cycle: G_1/S and G_2/M (21). It is known that cyclin E represents the key molecule of the G1/S checkpoint. The binding of endogenous CDKis, such as p21 and p27, regulates the activity of the complex cyclin E-CDK2 (22). Furthermore, the induction of p21 and p27 arrests cell cycle in the G_1 phase, thus inhibiting the cells from entering the S phase for replication (23). It is known that cyclin E overexpression can promote cancer progression, whereas its downregulation, by limiting cell cycle progression to the G_0/G_1 phase, can decrease and inhibit tumor cell proliferation (24,25). Similarly, p21 and p27 act as tumor suppressors by controlling cell cycle progression and cell proliferation (26-28); decreased expression levels of p21 and p27 have been detected in various types of human cancer (29-31). Finally, the efficacy of GEOCYDO in Mes2 cells that do not express p21, can be ascribed to the activity of p27 that regulates cell cycle progression through decreasing p21 expression (32).

The ability of GEOCYDO to induce G_1/S cell cycle arrest was confirmed by protein analysis, showing a downregulation in cyclin E, cyclin A and cyclin B1 expression, and a concomitant activation of p21 and p27. A hallmark of cancer is represented by uncontrolled and rapid cell division (2); therefore, the inhibition of cell cycle progression may be a powerful anticancer approach.

Natural marine compounds are known to exert anticancer activity *in vitro* and *in vivo* (33,34), as well as in clinical settings (35), by acting on the cell cycle (36). Several extracts have been described to induce cell cycle perturbations in various tumor cell lines. Extracts from the *Lissodendorix* sponge have been described to act by preventing microtubule assembly (37-39). The antiproliferative activity of an extract from the *Negombata magnifica* sponge has been related to specific G_0/G_1 and G_2/M cell cycle block (40). Furthermore, marine sponge compounds derived from *Jaspis stellifera* and *Monanchora viridis* have been reported to induce cell cycle arrest through the reduction of cyclin D1 expression (2,41). These findings are in agreement with the present findings, which demonstrated that GEOCYDO blocked the transition between phase G_1 and S, as indicated by the decrease in the expression levels of cyclin E, the key protein that regulates this passage.

Finally, to the best of our knowledge, the preliminary results from spectroscopic analysis indicated for the first time that GEOCYDO contained different active metabolites. The results are particularly promising since, to date, the knowledge of secondary metabolites derived from members of the genus *Geodia* (class, Demospongiae; family, Geodiidae) remains limited. The bioactivity of GEOCYDO was initially tested using the TE, after which the TE was fractionated and the different fractions were analyzed to identify the active fraction; the results revealed that fraction C was responsible for the bioactivity of GEOCYDO.

Molecular networking analysis of the bioactive GEOCYDO extract revealed that it may contain different molecules, such as nucleosides and amino acids, which are currently providing lead compounds for new drugs as main constituents (9). In particular, previous studies have reported that different nucleosides exert antiviral, anticancer and hypertensive effects (42,43); and that some amino acids have a high specificity against cancer cells (44). Moreover, GEOCYDO was shown to contain 3-hydroxyquinaldic acid, a chromophore present in natural antitumor agents that is required for DNA intercalation being able to binds duplex (45). In addition, the SPE fraction C exerted the strongest activity on mesothelioma cancer cell lines, as revealed by the IC_{50} value, suggesting this fraction may be enriched in a specific molecule that is responsible of the observed effect. Although fractionation of the extract highlighted the general characteristics of the compounds that are likely responsible for the activity of the extract, fraction C is still a complex mixture of metabolites; therefore, further chemical purifications are required to isolate and characterize the active compound.

The present results are novel since, despite the large number of marine compounds used as drug candidates in various types of cancer, to the best of our knowledge, none have assessed their effects on mesothelioma. The results of the present study also suggested that new molecules from marine organisms could be further investigated for novel treatment of this type of cancer, which is characterized by high chemoresistance.

In conclusion, GEOCYDO extract from the marine sponge *G. cydonium* could be considered a novel candidate as a potential antitumor drug for human malignant mesothelioma. This is a very important step in development of alternative and more effective therapies to cure mesothelioma, considering that to date the standard therapies, including chemotherapy, surgery and radiotherapy, have produced unsatisfactory outcomes (46).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SC, MC, SF and AF conceptualized the study. FDM, RC, MA and GF performed cellular and molecular biology experiments. GN, AF, NR and RE performed chemical extraction. FDM, RC, MA, GF, NR and RE performed data analysis. SC, MC, NR, RE, FDM and RC were responsible for original draft preparation. FDM, RE, RC, GF, MA, NR, GN, AF, SF, SC and MC confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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