Molecular Cancer Therapeutics

2 Q1
3 A Tricin Derivative from *Deschampsia Antarctica* 4 Q2 Desv. Inhibits Colorectal Carcinoma Growth and 5 Liver Metastasis through the Induction of a 6 Q3 Specific Immune Response

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11 Abstract

12 In colorectal carcinoma patients, distant metastatic disease is 13 present at initial diagnosis in nearly 25% of them. The majority of 14 patients with metastatic colorectal carcinoma have incurable dis- 15 ease; therefore, new therapies are needed. Agents derived from 16 medicinal plants have already demonstrated therapeutic activities 17 in human cancer cells. Antartina is an antitumor agent isolated 18 from *Deschampsia antarctica* Desv. This study aimed to evaluate the 19 antitumor properties of Antartina in colorectal carcinoma models. 20 We used human and murine colorectal carcinoma cell lines for 21 investigating proliferation, apoptosis, and cell-cycle effects of 22 Antartina therapy *in vitro*. Avatar and immunocompetent colorec- 23 tal carcinoma animal models were applied for evaluating the 24 effects of Antartina *in vivo*. Immune response against colorectal 25 carcinoma model was investigated using CTL assay, analyzing 26 dendritic cell activation and intratumor T-cell subpopulation, and 28 by tumor rechallenge experiments. Antartina inhibits *in vitro* 29 human colorectal carcinoma cell proliferation; however, *in vivo* 30 experiments in Avatar colorectal carcinoma model Antartina dis- 31 play a limited antitumor effect. In an immunocompetent colorec- 32 tal carcinoma mice model, Antartina potently inhibited tumor 33 growth and liver metastases, leading to complete tumor regressions 34 in >30% of mice and increased animal survival. In addition, 35 Antartina induced a potent specific cytotoxic T-cell response 36 against colorectal carcinoma and a long-lasting antitumor immu- 37 nity. Interestingly, Antartina increased tumor immunogenicity and 38 stimulated dendritic cell activation. No toxic effects were observed 39 at the doses employed. Our findings showed that Antartina has the 40 ability to induce antitumor immunity against colorectal carcinoma 41 and can be used to develop new tools for the treatment of colorectal 42 carcinoma. *Mol Cancer Ther; 1–11. ©2018 AACR.*

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44 Introduction

45 Colorectal carcinoma is a major cause of cancer-related mor- 46 bidity and mortality worldwide and is responsible for nearly 47 700,000 deaths each year (1). For metastatic disease, surgery is 49 a potential curative option, but less than 20% to 30% of patients 50 are suitable for resection due to clinical or technical causes, 51 including extrahepatic disease (2). In the past few years, multi- 52 disciplinary approaches such as chemotherapy regimens, radio- 53 embolization, and targeted therapies have been applied to 54 improve patient survival, but as noted above still remains signif- 55 icant mortality (3). Therefore, new therapeutic therapies are 56 needed for patients with a more advanced stage of the disease (4).

57 With its unique weather and environmental characteristics, 58 Antarctica is home to an extraordinary variety of extremophile 59 organisms, including vascular plants such as *Deschampsia* 60 *antarctica* (5). There are several examples of novel psychrophilic 61 enzymes and new molecules isolated from organisms found in 62 Antarctica with potential uses in medicine (6). In particular, 63 *Deschampsia antarctica* is able to tolerate high UV exposure due 64 to the production of secondary metabolites as photoprotector 65 agents, especially flavonoid-like molecules (7, 8). Tricin, a flavo- 66 noid type compound from the secondary metabolic pathways in 67 plants, has importance to plant growth by protecting against 68 disease, weeds, and microorganisms (9). Tricin is present in 69 herbaceous and cereal plants and exists as free tricin and its 70 derivatives, such as tricin-glycosides (9). The flavone tricin and 71 their derivatives have been shown to exhibit antiproliferative 72 activity against several human cancer cell lines, cancer chemo- 73 protective effects in the gastrointestinal tract of mice, and

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antioxidative effects (10–13). The reported antitumor activities of flavonoids involve the inhibition of proliferation and induction of apoptosis, suppression of protein tyrosine kinase activity, and antiangiogenic effects (14). However, little is known about the role of tricin derivatives and its bioactivity as anticancer molecule. Antartina (tricin 7-O-beta-D glucopyranoside) was isolated from the Antarctic plant *Deschampsia antarctica* and has been previously reported from other plants (15, 16). Because of low natural availability, a synthesis was conducted, and herein, we report on the antitumor activity of Antartina produced synthetically.

In this work, we show that Antartina induces a potent and specific immune response against colorectal carcinoma in mice. Therefore, Antartina represents a promising therapeutic agent for colorectal carcinoma patients.

Materials and Methods

Antartina

Tricin 7-O-β-D-glucopyranoside (initially called peak 10; molecular formula: $C_{23}H_{24}O_{12}$; CAS no. 32769-01-0, purity >99.2%) was detected and isolated from aqueous extracts of *Deschampsia antarctica Desv*. (*Poaceae*). Synthetic Antartina was supplied by the Centro de Química Aplicada y Biotecnología (CQAB), Universidad de Alcalá, Alcalá de Henares (Madrid, Spain). To determine the structure of Antartina, a yellow amorphous solid, encoded as peak 10, was used, obtained from aqueous extract MCC12-034-03 (JON 2009) according to methodology developed by CQAB, by solid phase extraction and then semipreparative high-performance liquid chromatography (HPLC). The mass spectrum (MS) analysis using the internal CQAB method confirms that the purity of the isolated sample was greater than 99.2% (detection at 254 nm). The MS, in electrospray positive mode (ESI^+), gives rise to a peak with a mass/charge ratio (m/z 493), whereas in electrospray negative mode (ESI^-) an m/z signal 491. This indicates a molecular mass for the product of 492 Da. A high-resolution time-of-flight mass analysis gave m/z 492.1268 (Calc. 492.1267) corresponding to a formula of $C_{23}H_{24}O_{12}$. The ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses validate that the product is the 5-hydroxy-2-(4-hydroxy-3,5-dimethoxy-phenyl)-7-[(2S,3S,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl] oxychromen-4-one. Then, a synthetic method has been developed, and all the Antartina samples used in the tests were of synthetic origin.

Cell lines

Mouse CT26 tumor cell line, an undifferentiated murine colorectal carcinoma cell line established from a N-nitroso-N-methylurethan–induced transplantable tumor in BALB/c (H-2d) mice and BNL cells (a hepatoma cell line) was kindly provided by Prof. Jesús Prieto, University of Navarra (Pamplona, Spain). We have maintained these cell lines since 2007, and they have been tested for mycoplasma every time that they were thawed and cultured by the PCR-based detection procedure involving three steps: cell culture supernatant collection, DNA isolation, and PCR. Human cell lines were purchased from ATCC: LoVo (ATCC-CCL229); ASG (ATCC-CCRL-79); and lung fibroblasts Wi38 (ATCC-CCL75). No authentication was done by the authors.

All the cell lines were tested for mycoplasma as we described above and cultured in complete DMEM (GIBCO, Thermo Fisher Scientific; 2 mmol/L glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin; and 10% heat-inactivated FBS; GIBCO, Thermo Fisher Scientific) and incubated at 37°C in a 5% CO_2 humidified atmosphere. For *in vitro* assays, cells were thawed and cultured generally for 48 hours, until 90% to 100% confluence. Then, cells were dissociated using tripsin, passed, and cultured for another 48 hours. For *in vivo* experiments, cells were cultured at least for one week with two or three passages until inoculation.

Drugs

Antartina was dissolved in 0.1% DMSO and sterile water and injected intraperitoneally at the doses and the schedule indicated or used for *in vitro* assays at different concentrations (0.1, 1, 10, 100, and 1,000 μg/mL). Tricin was dissolved in 0.3% DMSO and sterile water and injected intraperitoneally. Fluorouracil Rontag (5-FU) 500 mg was diluted in saline at indicated concentrations for *in vitro* assays or injected intraperitoneally for *in vivo* experiments.

In vitro experiments

Cell viability assays. LoVo, ASG, Wi38, and CT26 cells were plated onto 96-well plates at a density of 5×10^3 cells per well and cultured with DMEM 0.1% DMSO or Antartina at different doses. Forty-eight and 72 hours after treatment, viability was determined by the MTS assay (Promega). The plates were incubated for 4 hours; the absorbance of each well was read at 490 nm. All assays were performed in quadruplicate, and each assay was repeated at least twice. Morphologic features associated with apoptosis were analyzed by acridine orange (AO) and ethidium bromide (EB) staining. CT26 cells were treated with 10 μg/mL Antartina, and 24 or 72 hours later, cells were resuspended in the dye mixture (100 μg/mL AO and 100 μg/mL EB in PBS) and visualized by fluorescence microscopy (Nikon Eclipse E800).

Cell-cycle analysis. CT26 cells (1×10^6) cultured with DMEM or Antartina were collected, washed in PBS, and fixed in a mixture of ice-cold 70% (v/v) ethanol, FBS, and distilled water. Fixed cells were centrifuged and stained with propidium iodide (PI) solution (50 μg/mL PI, 180 U/mL RNase). DNA content was determined using a FACS Accuri 6 laser flow cytometer (Becton Dickinson).

In vivo experiments

Avatar model. Four- to 6-week-old female athymic nude-Foxn1nu, (nu/nu) mice were purchased from Envigo. Animals were maintained at the Spanish National Cancer Research Centre Animal Facility (awarded with the AAALAC accreditation) in accordance with the guidelines stated in the International Guiding Principles for Biomedical Research Involving Animals, developed by the Council for International Organizations of Medical Sciences. All animal experiments were approved by the Competent Authority of Comunidad de Madrid (project PROEX 104/16). We attempted to establish an Avatar model from each of the patients following the methodology previously published by Hidalgo and colleagues (17, 18).

Briefly, a tumor specimen obtained by a tumor biopsy was transplanted and propagated in nude mice. Avatar models were mostly generated by specimens obtained from fresh biopsies of metastasis, as they were generally more accessible than the primary tumors and generally represent a more advanced tumor clone with additional driver/aggressive mutations. Once the tumor specimen was in an exponential growth phase, cohorts of mice with tumor sizes of 100 to 300 mm^3 were randomized to the

treatment groups indicated in the figures. Animals were treated with Antartina or 0.1% DMSO in sterile water intraperitoneally (Antartina; 5 mg/kg i.p., day 8; 3 times a week during 3 weeks). Tumor growth was assessed 3 times a week by caliper measurement.

Subcutaneous colorectal carcinoma model. Six- to 8-week-old male BALB/c mice were purchased from Fundación Balseiro (Buenos Aires, Argentina). Animals were maintained at our Animal Resources Facilities in accordance with the experimental ethical committee and the NIH (Bethesda, MD) guidelines on the ethical use of animals. The Animal Care Committee from School of Biomedical Sciences, Austral University (Buenos Aires, Argentina), approved the experimental protocol.

CT26 cells were injected at a dose of 5×10^5 cells subcutaneously into the right flank of BALB/c mice (day 0). Tumors were allowed to reach approximately 90 mm^3 in size before treatment. Animals were distributed in different groups and then treated with 0.1% DMSO in sterile water intraperitoneally (control group, $n = 7$), or Antartina (5 or 50 mg/kg i.p., day 8, $n = 9$), tricin (50 mg/kg $n = 8$), or 5-FU (50 mg/kg; $n = 6$) 3 times a week during 3 weeks. Tumor growth was assessed by caliper measurement. Tumor volume was calculated using the following formula: tumor volume (mm^3) = length $\times$ $(\text{width})^2/2$ (19).

Liver metastatic colorectal carcinoma model. To study the effects of Antartina in a more aggressive intrahepatic tumor model (20), BALB/c mice received an intrahepatic inoculation of 5×10^5 CT26 cells (day 0). At day 8, mice were distributed in experimental groups and treated with 0.1% DMSO in sterile water intraperitoneally (control group, $n = 6$) or Antartina (5 mg/kg i.p., $n = 8$) 3 times a week during 3 weeks. At day 18, animals were sacrificed and the volume of metastatic nodules was measured with caliper.

Adoptive T-cell therapy. On day 8, CT26 tumor-bearing mice received a single injection of 1×10^6 $CD3^+$ T cells isolated by magnetic cell sorting as it was described below, and as we previously reported (21). We evaluated the effects of ATC with Antartina alone, and untreated CT26 tumor-bearing mice were used as control. Tumor growth was assessed by caliper measurement.

Long-term protection study. To evaluate protective immunity, animals that were free of tumor at 3 weeks after complete regression of primary tumors (7 weeks after first tumor inoculation) were challenged with 5×10^5 CT26 cells on the left flank.

Ex vivo experiments

Histology. Tumor samples from experimental groups were obtained 10 and 15 days after treatment and fixed in 10% phosphate-buffered formalin. Five-micrometer sections from paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) for histologic examination.

Proliferation assay. To measure the proliferation of splenocytes cultured with 0.1 to 10 μg/mL of Antartina, [^{3}H] thymidine incorporation assay was performed. For splenocyte isolation, spleens from healthy mice were obtained, mechanically disrupted, and red blood cells were lysed. *In vitro* stimulation was performed in RPMI medium for 48 hours in 96-well plates containing 5×10^5 splenocytes and Antartina or 10 μg/mL of concanavalin A as control of splenocytes proliferation. Each sample was analyzed 6-fold. Briefly, 5 μCi/mL [methyl-3H] thymidine (specific activity 20 Ci/mmol; PerkinElmer) was added to culture and incubated for 24 hours. Cells were harvested and radioactivity was determined by using a liquid scintillation counter (Beckman LS 6500).

Flow cytometry analyses. Tumor samples were treated with 0.5 mg/mL Collagenase I at 37°C for 45 minutes and washed with PBS 1% BSA to obtain single-cell suspensions by mechanical disruption. Then, tumor cells were stained with PECy5 anti-CD4 (BD Biosciences) and Alexa Fluor 488 anti-CD8 (BD Biosciences) and their respective control isotypes. Staining of generated dendritic cells (DC) was carried out using different conjugated antibodies as follows: anti-CD11c, anti–MHC-II, anti-CD80, and their respective control isotypes (BD Biosciences). Cells were analyzed by flow cytometry (FACSAria, BD Biosciences) and the Cyflogic v. 1.2.1 software was used.

Tumor lysates. Seven days after treatment, tumor samples were obtained and frozen at −80°C; then, samples were disrupted by 5 freeze–thaw cycles. To remove large debris, tumor lysates were centrifuged at 300 rpm for 10 minutes. The supernatant was collected and filtered (0.2 μm). The protein concentration of the lysate was determined by Bradford assay. Tumor lysates were aliquoted and stored at −40°C until use.

Bone marrow–derived DC generation. Bone marrow–derived DCs from untreated or Antartina treated tumor-bearing BALB/c mice were generated as described previously (22). Briefly, 10^6 bone marrow cells/mL were plated into 6-well plates and cultured for 7 days with 20 ng/mL of GM-CSF (PeproTech) at 37°C with 5% CO_2. Medium was replaced on days 3 and 5 of culture. At day 7, DCs were centrifuged and pulsed with whole tumor lysates (200 μg/10^6 cells/ml) at 37°C for 18 hours. In addition, bone marrow DCs were generated in the presence of 10 μg/mL Antartina and cultured during 7 days to characterize their status of maturation by flow cytometry.

Peripheral blood mononuclear cell–derived DC. Peripheral blood mononuclear cells from healthy donors and patients were isolated by Ficoll–Paque gradient (Sigma). Cells were plated into 6-well plates for 2 hours. Then, adherent cells were cultured for 7 days in RPMI1640 medium (Invitrogen), containing 10% FCS (Invitrogen), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, human recombinant GM-CSF (Bioprofarma, Growgen, 350 ng/mL), and 2-β-mercaptoethanol (0.05 mol/L). Cells were preconditioned with 10 μg/mL Antartina and stained with anti-human CD11c-PE, anti-human MHC-II-FITC, and anti-human CD80-APC (BD Biosciences) by flow cytometry at day 7.

Isolation of $CD3^+$ T lymphocytes. To evaluate specific T-cell response induced by Antartina, CT26 tumo- bearing mice were treated as described above. Splenocytes from cured mice were isolated and pooled; 2×10^6 cells/mL were cocultured with mitomycin C–treated CT26 cells (2×10^5/mL) in a 24-well plate (1 mL/well) with mouse recombinant IL2 (10 IU/mL). Seven days later, viable cells were harvested and washed, adjusted to 2×10^6/mL, and cocultured again with mitomycin C–treated CT26 cells with 10 IU/mL of IL2. The cytotoxic activity of harvested cells was confirmed with the LDH Cytotoxicity Detection

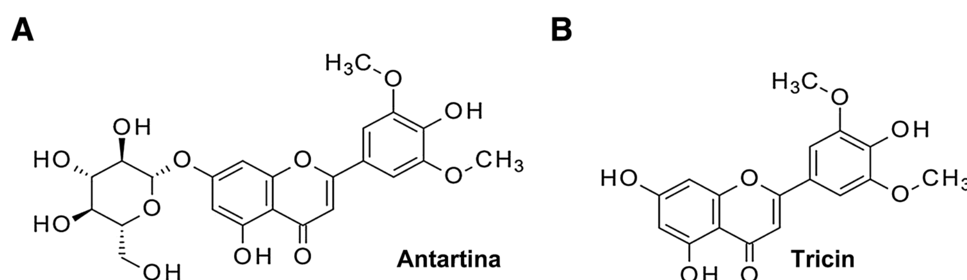

Figure 1.
Antartina. **A,** Structure of Antartina (tricin 7-O-β-D-glucopyranoside). **B,** Structure of tricin.

Kit (Sigma-Aldrich). On day 14, viable cells were used for isolation of $CD3^+$ T lymphocytes using anti-mouse $CD3^+$ MicroBeads and magnetic cell sorting following the manufacturer's recommendations (Miltenyi Biotec).

Cytotoxicity assay. Viable splenocytes from control or treated mice (1×10^7) were stimulated *in vitro* with mitomycin C–treated CT26 cells (1×10^6 cells/well in 24-well plates). On day 5, cells were harvested and washed, adjusted to 2×10^6/mL, and added to 96-well plates (effector cells). To determine specific cytotoxic T lymphocyte (CTL) cytotoxicity activity, CT26 and BNL cells were used as target at 2×10^5/mL. After incubation for 4 hours at 37°C, plates were centrifuged and cell-free supernatants were obtained. Levels of released LDH were evaluated with the LDH Cytotoxicity Detection Kit and expressed as percentage of lysis.

Toxicity studies. BALB/c mice untreated or treated with Antartina (5 and 50 mg/kg) were used to assess toxicology. Mice were observed for more than 30 days. Animal weight was registered and aspartate aminotransferase and alanine aminotransferase levels were measured by standard colorimetric methods. Healthy mice or mice receiving Antartina were sacrificed to collect liver, spleen, and kidney samples. Paraffin-embedded tissues were stained with H&E for pathologic analysis.

Statistical analysis

All experiments were repeated at least three times. Values were expressed as the mean ± SEM. The Student *t* test, Mann–Whitney test, or ANOVA with Tukey test were used to evaluate the statistical differences between two groups or more than two groups. Mice survival was analyzed by a Kaplan–Meier curve. A P value of <0.05 was considered statistically significant. Prism software (GraphPad) was employed for the statistical analysis.

Results

Antartina isolation and identification

Antartina has been detected and obtained by isolation using semipreparative HPLC from aqueous extracts of *Deschampsia antarctica* Desv. (*Poaceae*; Peak 10, Fig. 1A). The purity and structural identification of the isolated peak 10 was performed by NMR and analyzed by mass spectrometry. Peak 10, isolated from *Deschampia antarctica* extract, is tricin 7-O-β-D-glucopyranoside, also denominated 5-hydroxy-2- (4-hydroxy-3,5-dimethoxy-phenyl) -7 - [(2S, 3S, 4R, 5S, 6R) -3,4,5-trihydroxy-6- (hydroxymethyl) tetrahydropyran-2-yl] oxychromen-4-one. Then, tricin 7-O-β-D-glucopyranoside or Antartina was obtained following the synthetic method indicated in Supplementary Material.

Antartina reduced human cancer cell viability *in vitro* and has modest effects *in vivo* in the Avatar model

It has been reported that tricin, a natural flavone, and its derivatives exert antioxidative effects and exhibit antiproliferative activity in tumor cells. Therefore, we decided to assess the effects of Antartina on *in vitro* cultured human colorectal carcinoma cell line (LoVo). Figure 2A shows that Antartina inhibited tumor cell viability in a dose-dependent manner. Antartina also decreases the viability of ASG gastric carcinoma cells, whereas at the same concentrations, it has no effect on normal human fibroblasts (Wi38 cells; Fig. 2A). Taking into consideration the *in vitro* cytotoxic activity of Antartina against human colorectal carcinoma cells, we decided to examine the capability of Antartina to reproduce its effects in *in vivo* model using small fragments of fresh surgical specimens of colorectal carcinoma liver metastasis in the Avatar model, an immunodeficient mice model. To this end, nu^+/nu^+ mice were subcutaneously inoculated with a piece of approximately 80 mm^3 of tumor sample from patients with colorectal carcinoma. Over the course of 40 days, we observed that tumor-bearing mice treated with 5 mg/kg Antartina showed a mild inhibition of tumor growth in comparison with control group (Fig. 2B).

Effects of Antartina on immunocompetent colorectal carcinoma murine models

To assess whether the limited antitumor effect of Antartina was related to the absence of the adaptive immune system arm, we tested the efficacy of Antartina in immunocompetent murine models of colorectal carcinoma. For this purpose, we first incubated *in vitro* CT26 cells with increasing doses of Antartina for 48 and 72 hours. We observed that 10 μg/mL Antartina significantly inhibited CT26 cell survival at 48 hours ($P < 0.05$, Fig. 3A). In addition, cell viability was reduced at 72 hours to approximately 60% at 10 μg/mL of Antartina, and more than 15% at the dose of 1 μg/mL ($P < 0.001$ and $P < 0.05$, respectively). However, when compared with 5-FU, Antartina exhibited a lower capacity to decrease CT26 cell viability (Fig. 3B). It has been previously observed that tricin showed capability to block cell-cycle progression of some cancer cell lines (10, 23). We examined whether Antartina might affect cell cycle and observed that CT26 cells were arrested at G_2–M phase both at 48 and 72 hours after incubation with 10 μg/mL of Antartina ($P < 0.05$; Fig. 3C). Similar results were obtained when cell-cycle analysis was performed 72 hours after treatment with 5-FU ($P < 0.05$, Fig. 3D), whereas at 48 hours, the antimetabolite induced a clear arrest at the S-phase. Importantly, Antartina has the ability to induce apoptosis on CT26 cells when cells were incubated with 10 μg/mL of Antartina for 72 hours ($P < 0.05$, Fig. 3E).

In view of the modest cytotoxic effect in CT26 cells observed *in vitro*, we next tested Antartina activity *in vivo*. To this end, BALB/c

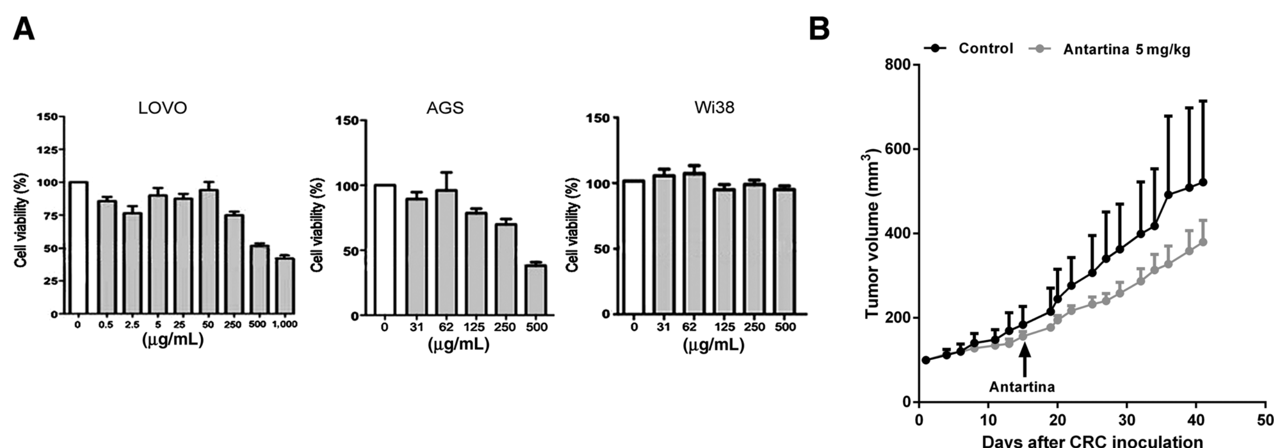

Figure 2.
Antiproliferative effects of Antartina on human cancer cells. **A,** Antartina reduced the viability of LoVo (colorectal) and ASG (gastric) carcinoma cells lines determined by MTS assay at 72 hours; human Wi38 fibroblasts remained viable at the same doses. Percentages of viable cells ± SD are presented for each cell line. **B,** Nu/nu mice bearing subcutaneous palpable human tumors were untreated ($n = 4$) or treated intraperitoneally with 5 mg/kg of Antartina ($n = 5$) 3 times a week. CRC, colorectal carcinoma. Mean of tumor sizes ±SD are presented for each experimental group.

mice were subcutaneously inoculated with CT26 tumor cells, and treatments were initiated when tumors reached a tumor volume approximately 90 mm^3. Over the course of 30 days, CT26 tumor-bearing mice treated with Antartina showed a significant inhibition of tumor growth in comparison with the control group (Fig. 4A). Importantly, survival rate of mice receiving Antartina was significantly increased compared with controls ($P < 0.01$; Fig. 4B). Then, we compared the *in vivo* efficacy of Antartina with a similar molecule tricin (Fig. 1B) and with 5-FU in the CT26 colorectal carcinoma model. Antartina was significantly superior in terms of tumor growth inhibition in comparison with tricin and 5-FU (Supplementary Fig. S1).

We also challenged Antartina anticancer activity in a metastatic colorectal carcinoma model. For this purpose, CT26 cells were injected directly into the liver of mice by laparotomy (day 0) and then treated with Antartina at a dose of 5 mg/kg on day 8. Figure 4C showed mean tumor size at day 18 after CT26 cell inoculation. Interestingly, Antartina exerted a potent antitumor effect in comparison with control group (mean of tumor volume 955 ± 275 mm^3 vs. 1,508 ± 204 mm^3, respectively); in addition, more than 60% of Antartina-treated animals showed a reduction in metastases growth. More importantly, survival of mice treated with Antartina was superior in comparison with controls ($P < 0.01$, Fig. 4D).

Then, we decided to study higher doses of Antartina. To this end, CT26 colorectal carcinoma cells were subcutaneously injected into BALB/c mice (day 0). On day 8, nodules reaching 90 mm^3 in size received 50 mg/kg of Antartina or vehicle. A potent tumor volume reduction was obtained with Antartina, and complete tumor regression was achieved in more than 30% of mice (3/9; Fig. 5A). Tumor progression was evaluated at different time points (10 and 15 days after treatment) as shown in Fig. 5B. Microscopic examination of tumor sections showed extensive areas of necrosis and marked mononuclear cell infiltration in tumors treated with Antartina (Fig. 5D). In addition, animal survival was significantly increased in Antartina-treated mice compared with the control group ($P < 0.01$, Fig. 5C).

Antartina therapy generates a potent antitumor immune response

In view of the marked presence of mononuclear cells infiltrated into Antartina-treated tumors, we investigate the effect of Antartina on immune cells. When splenocytes from healthy mice were incubated for 48 hours with 0.1 to 10 μg/mL of Antartina, we observed a significant induction of splenocytes proliferation (Fig. 6A). Remarkably, Antartina (10 μg/mL) significantly inhibited CT26 cell survival (Fig. 3A). These data suggest that our molecule could be able to inhibit tumor growth without distressing normal cells but also inducing the activation of the immune system. We then analyzed the prevalence of T lymphocytes in tumor samples by flow cytometry and detected an increased proportion of tumor-infiltrating CD4$^+$ and CD8$^+$ T cells in Antartina-treated mice in comparison with controls ($P < 0.05$; Fig. 6B).

To evaluate whether Antartina was able to enhance tumor immunogenicity, we analyzed the activation status of DCs. Tumor lysates derived from CT26-bearing mice were used to pulse bone marrow–derived DCs and to determine changes in the maturation status of DCs by the expression levels of CD11c, the class II MHC and CD80 molecules. As shown in Fig. 6C, we observed that Antartina induced a higher activation profile in DCs based upon the following: when DCs derived from untreated CT-26 tumor-bearing mice were treated with tumor lysate derived from Antartina-treated mice, a greater increase in levels of costimulatory molecules (CD11c, MHC, and CD80) was observed than in DCs from untreated CT-26 tumor-bearing mice treated with tumor lysate from untreated mice (Fig. 6C; $P < 0.05$, Mann–Whitney test). These results may reflect the capacity of Antartina to increase tumor immunogenicity. In addition, when cultured murine and human DCs were incubated with Antartina, a high activated profile was observed, suggesting that Antartina could be used as an adjuvant for activation of DCs as anticancer vaccines (Supplementary Fig. S2).

On the other hand, Antartina was found to elicit a potent activation of specific CTLs against CT26 cells (Fig. 6D; $P < 0.001$); no CTL activity was observed against hepatocellular carcinoma cells (BNL cells) *in vitro*. Interestingly, when specific

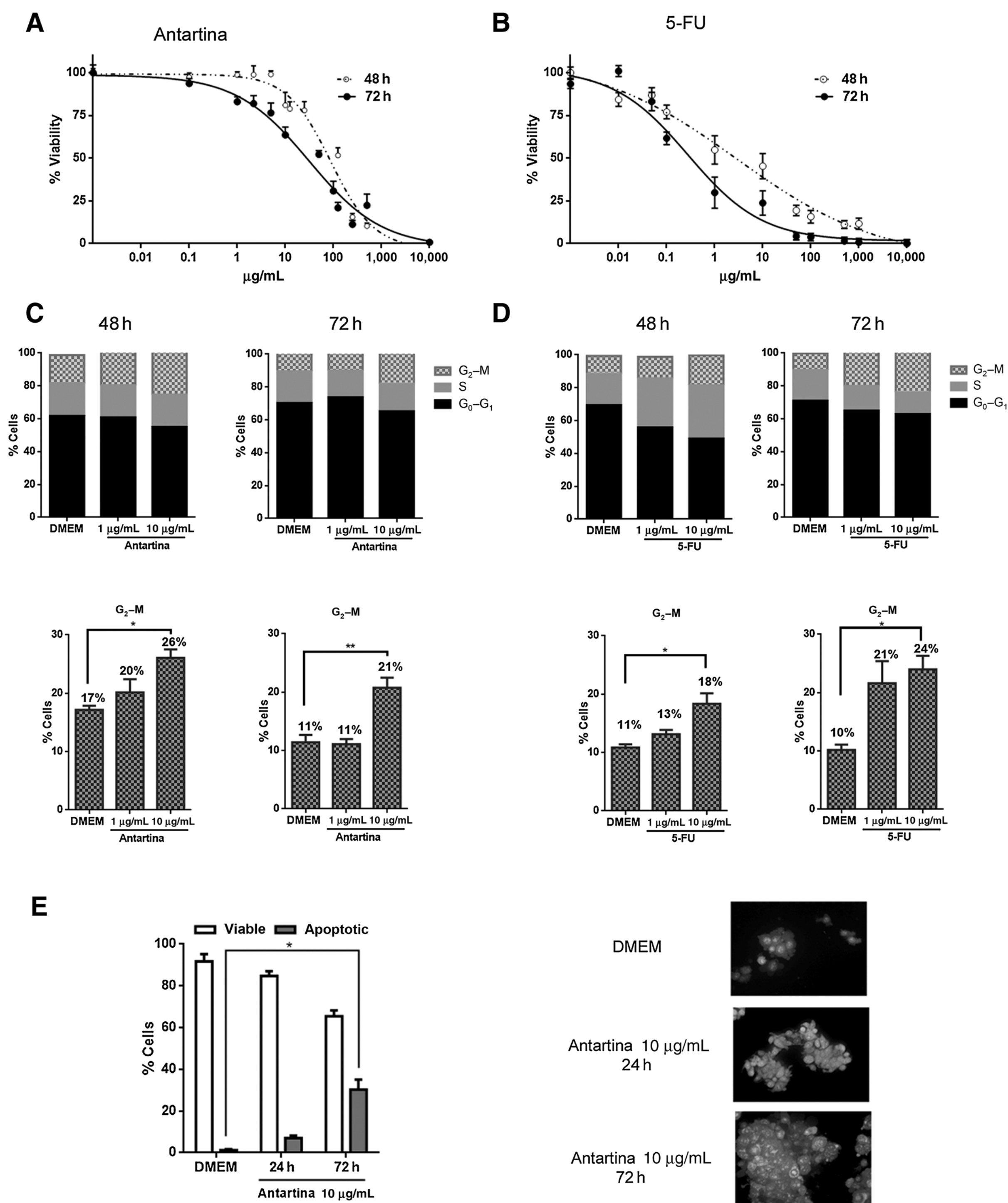

Figure 3.

Antartina inhibits murine CT26 colorectal carcinoma cell growth *in vitro*. **A,** CT26 cells were incubated with DMEM or with increasing concentrations of Antartina during 48 or 72 hours. Cell viability was determined by MTS assay in 5 independent experiments. Percentages of viable cells ±SD are presented. *, $P < 0.05$ (DMEM 0.1% DMSO vs. 10 μg/mL Antartina 48 hours); ***, $P < 0.001$ (DMEM 0.1% DMSO vs. 10 μg/mL Antartina 72 hours); and *, $P < 0.05$ (DMEM 0.1% DMSO vs. 1 μg/mL Antartina 72 hours) ANOVA and Tukey test. **B,** The effect of Antartina was compared with 5-FU in their ability to inhibit colorectal carcinoma cells growth. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using ANOVA and Tukey test. **C,** Cell-cycle flow cytometry analysis of CT26 cells stained with propidium iodide at 48 and 72 hours; Antartina (10 μg/mL) incubation halted CT26 cells at the transition from G_2 to M-phase. **, $P < 0.01$ (DMEM 0.1% DMSO vs. 10 μg/mL Antartina 72 hours) or *, $P < 0.05$ (DMEM 0.1% DMSO vs. 10 μg/mL Antartina 48 hours) ANOVA and Tukey test. **D,** Cell-cycle analysis of 5-FU–treated cells. *, $P < 0.05$ (DMEM vs. 10 μg/mL 5-FU 48 and 72 hours) ANOVA and Tukey test. **E,** *In vitro* CT26 cell apoptosis was assessed at 10 μg/mL Antartina using acridin orange/ethidium bromide staining (right, magnification ×40). Bars represent the average of measures of each group ± SEM (left). *, $P < 0.05$ (DMEM 0.1% DMSO vs. 10 μg/mL Antartina 72 hours).

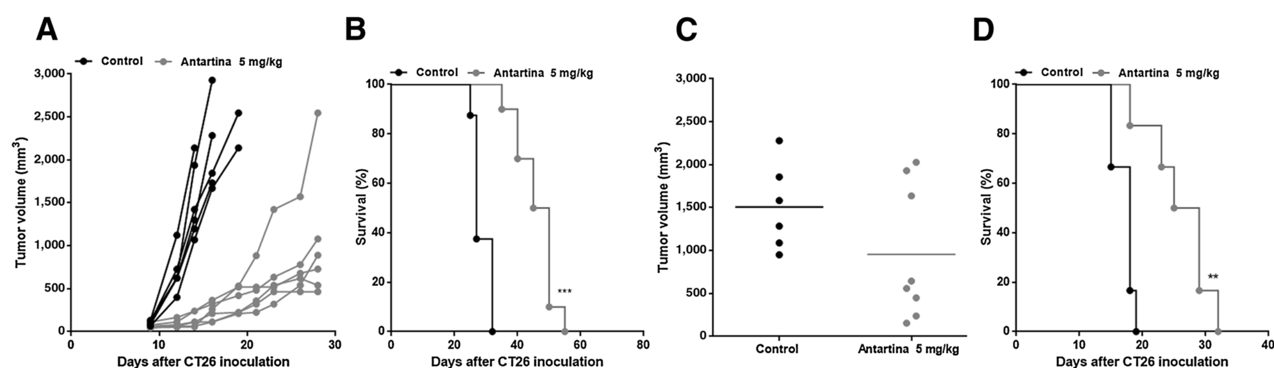

Figure 4.
Antitumor activity of Antartina in CT26 colorectal carcinoma tumor models *in vivo*. **A,** Subcutaneous model: BALB/c mice were subcutaneously injected with 5×10^5 CT26 cells into the right flank (day 0) and tumors reach 90 mm^3 before treatment was started. Animals were distributed in groups: control (0.1% DMSO in sterile water), Antartina (5 mg/kg i.p., day 8 three times a week, for 3 weeks). Data are expressed as tumor volume for each mice; unpaired *t* test. **, $P = 0.0009$ control versus Antartina. **B,** Animal survival (Kaplan–Meier, log-rank test, $P < 0.001$). **C,** Liver colorectal carcinoma metastatic model: BALB/c mice were injected with 5×10^5 CT26 cells directly into the liver by laparotomy (day 0). Animals were distributed into different groups: control, Antartina (5 mg/kg i.p., day 8 three times a week, for 3 weeks). Data are expressed as tumor volume; unpaired *t* test. *, $P = 0.0472$ control versus Antartina. **D,** Animal survival (Kaplan–Meier, log-rank test, $P < 0.001$).

CTLs were adoptively transferred into CT26 tumor-bearing mice, a significant inhibition of tumor growth was achieved compared with controls (Fig. 6E). Our results strongly suggest that the potent antitumor effect induced by Antartina is mediated, at least in part, by the induction of antitumor immunity against CT26 colorectal carcinoma. We next investigated whether Antartina can induce memory immune response; to this end, cured animals were rechallenged with CT26 colorectal carcinoma cells 3 weeks after complete tumor regression. Figure 6F shows that CT26 cells were rejected in all animals of Antartina group; on the contrary, tumors grew in all mice in the control group. These data indicate that Antartina has the ability to induce long-term protection against tumor recurrence.

Finally, we characterized the toxicology profile of Antartina therapy (at the doses of 5 and 50 mg/kg) in mice. We found that all animals remained healthy throughout the experimental protocol; body weight gain in all groups of mice was similar during the observed period. Moreover, Antartina was well tolerated with no evident signs of clinical and biochemical toxicity within the studied period of time (Supplementary Fig. S3).

Discussion

Despite progress due to the combination of chemotherapy and targeted therapies, such as bevacizumab and cetuximab/panitumumab, patients with metastatic colorectal cancer still have a low long-term survival (24, 25); thus, it is necessary to develop new nontoxic and effective therapies for advanced disease (4).

Because of low natural availability, the compound was generated synthetically, and herein, we report on the activity of this synthetic material, which we call Antartina. This study found that Antartina not only elicits a potent antitumor response in mice with colorectal carcinoma but also induces a systemic antitumor immunity. A tumor-specific T-cell response was generated, which was further exploited in adoptive T-cell therapy protocol with significant antitumor effects. We observed that the ability of Antartina to induce apoptosis of CT26 cells and to increase the activation of DCs is important to the tumor-specific immune response. Importantly, Antartina therapy was very well tolerated, and no toxic effects were observed.

Certain compounds derived from medicinal plants, such as flavonoids and isoflavonoids, have received much attention because of their protective effects observed in populations with low incidence of colorectal, breast, and prostate cancer (14). Their antitumor activity involves a number of mechanisms: flavonoids act on reactive oxygen species, on cell signal transduction pathways related to cellular proliferation by inhibiting the transcription factor NF-κB or by inducing apoptosis, and also on cell migration (26–29). In particular, their capacity to inhibit tyrosine kinase activity and to suppress metalloproteinases was associated with inhibition of tumor development (30, 31). In line with this, it has been recently reported that tricin 4′-O-(erythro and threo β-guaiacylglyceryl) ether induced apoptosis in colorectal, ovarian, and breast cancer cells through mitochondrial membrane potential loss and chromatin condensation (32). These *in vitro* effects were observed using similar dose concentrations as we applied in our work. However, when we compared similar doses of tricin *in vivo* in mice with colorectal carcinoma, antitumor effects were mild. This led us to speculate that the superior antitumor effect of Antartina in comparison with other flavonoids depends, at least in part, on the stimulation of the immune system. Dietary flavonoids could be considered as cancer-preventive agents, but little is known about their application in immunotherapy. It has been previously described that tricin might block cell-cycle progression in breast carcinoma cells (10). Similarly, we observed that Antartina was able to inhibit human and murine tumor cells viability in a dose-dependent manner, without affecting survival of normal cells such as fibroblasts and splenocytes.

It was previously observed that the polyhydroxylated flavonoid quercetin has the ability to inhibit cancer cell growth *in vitro* (31, 33). Kashyap and colleagues showed that the inhibitory effect on gastric and colorectal carcinoma cell proliferation induced by quercetin seems to be dependent on the interference with cell-cycle events (34). In agreement with this, Antartina affects the cell-cycle arresting CT26 cells at the G_2–M phase. Although the intrinsic mechanism behind this effect was not identified yet, we

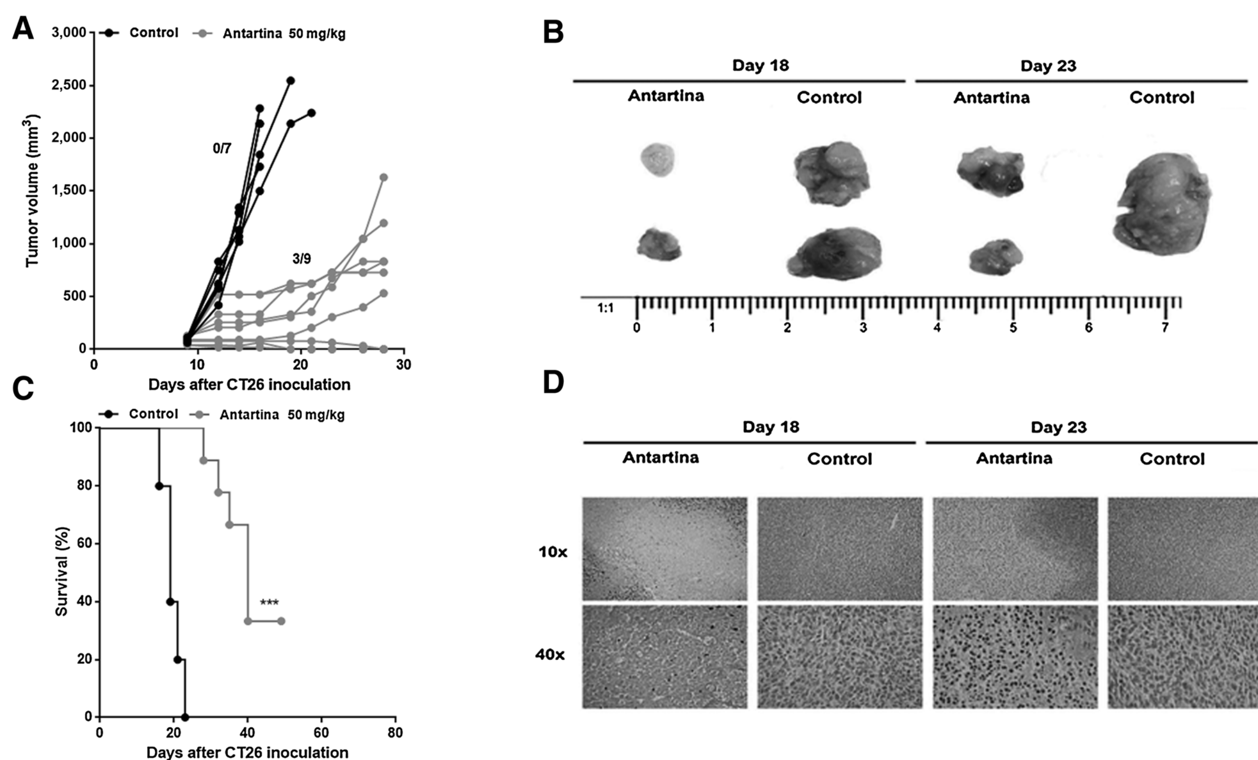

Figure 5.

Higher doses of Antartina exerted a more potent antitumor effect against CT26 colorectal carcinoma tumors *in vivo*. **A,** BALB/c mice were subcutaneously injected with 5×10^5 CT26 cells into the right flank (day 0) and tumors reach approximately 90 mm^3 before treatment was started. Animals were distributed in two groups: control and Antartina (50 mg/kg i.p., 3 times a week, during 3 weeks). Data are expressed as tumor volume; unpaired *t* test. ***, $P < 0.001$ control versus Antartina. We include the number of cured mice for each group (0/7 for control and 3/9 for Antartina). **B,** Representative macroscopic images of treated and untreated tumors at 18 and 23 days after CT26 cells inoculation. **C,** Animal survival (Kaplan–Meier, log-rank test, $P < 0.01$). **D,** Representative image of H&E-stained tumors from BALB/c mice treated or not with Antartina; Antartina-treated tumors showed intense mononuclear infiltrate and extensive areas of necrosis in comparison with control; magnification of tumor regions (×20). Scale bar, 50 μm.

showed that Antartina promotes apoptosis in colorectal carcinoma cells *in vitro*. Thus, our first results suggested the use of Antartina as a chemotherapeutic or chemopreventive agent, although it exerted a modest *in vitro* effect. The antitumor effects of Antartina were examined *in vivo* in immunocompromised nude mice using patient-derived xenografts (Avatar model). Unfortunately, the antitumor effect of Antartina was mild compared with controls. In contrast, when Antartina was tested in an immunocompetent murine colorectal carcinoma model, a potent volume reduction of subcutaneous tumors and also a significant inhibition in hepatic metastases growth was observed, suggesting that the limited antitumor effect of Antartina observed in nude mice was related to the lack of an efficient adaptive immune response.

As previously mentioned, flavonoids have been reported to have a wide range of biological activities (35), but information regarding immunostimulatory properties is very limited. It has been recently reported that salvigenin, a polyoxygenated flavone, stimulates the immune system in mice leading to the inhibition of tumor growth in a breast cancer model (36). The therapeutic efficacy of salvigenin involves the activation of splenocyte proliferation and the modulation of cytokine production of immune cells (36). Herein, we demonstrated that Antartina potently stimulated the immune system. Our hypothesis is that Antartina therapy modulated the tumor microenvironment in a way that cancer cells were more effective at inducing DC activation, and perhaps more potently to stimulate a specific CTL response against colorectal carcinoma cells. The immunomodulatory approach described in this work appears to be an attractive strategy to tip the balance for the generation of antitumor immunity. DC-based immunotherapy has been used to promote activation of nonfunctional DCs in patients with advanced cancer with the aim to stimulate a potent T-cell response (37). Part of the success is dependent on the generation in culture of mature DCs with high capacity to stimulate T cells. However, several DC-based immunotherapy strategies failed in the clinic because DC cultures generate immature DCs (38). In this study, we showed that murine and human DCs incubated with Antartina were activated, suggesting that Antartina could be employed as an adjuvant for DC activation. In our work, DCs obtained from Antartina-treated mice primed with tumor lysate from Antartina-treated mice showed higher levels of costimulatory molecules in comparison with DCs treated with tumor lysate from control mice. Therefore, it is possible to speculate that Antartina promotes immunogenic cell death (39) and that this may explain, at least in part, why Antartina therapy increased tumor immunogenicity. Although some chemotherapeutic agents can induce tumor-specific immune responses (e.g., mitoxantrone, 5-FU, camptothecin, and cisplatin; ref. 40), mainly through the induction of immunogenic

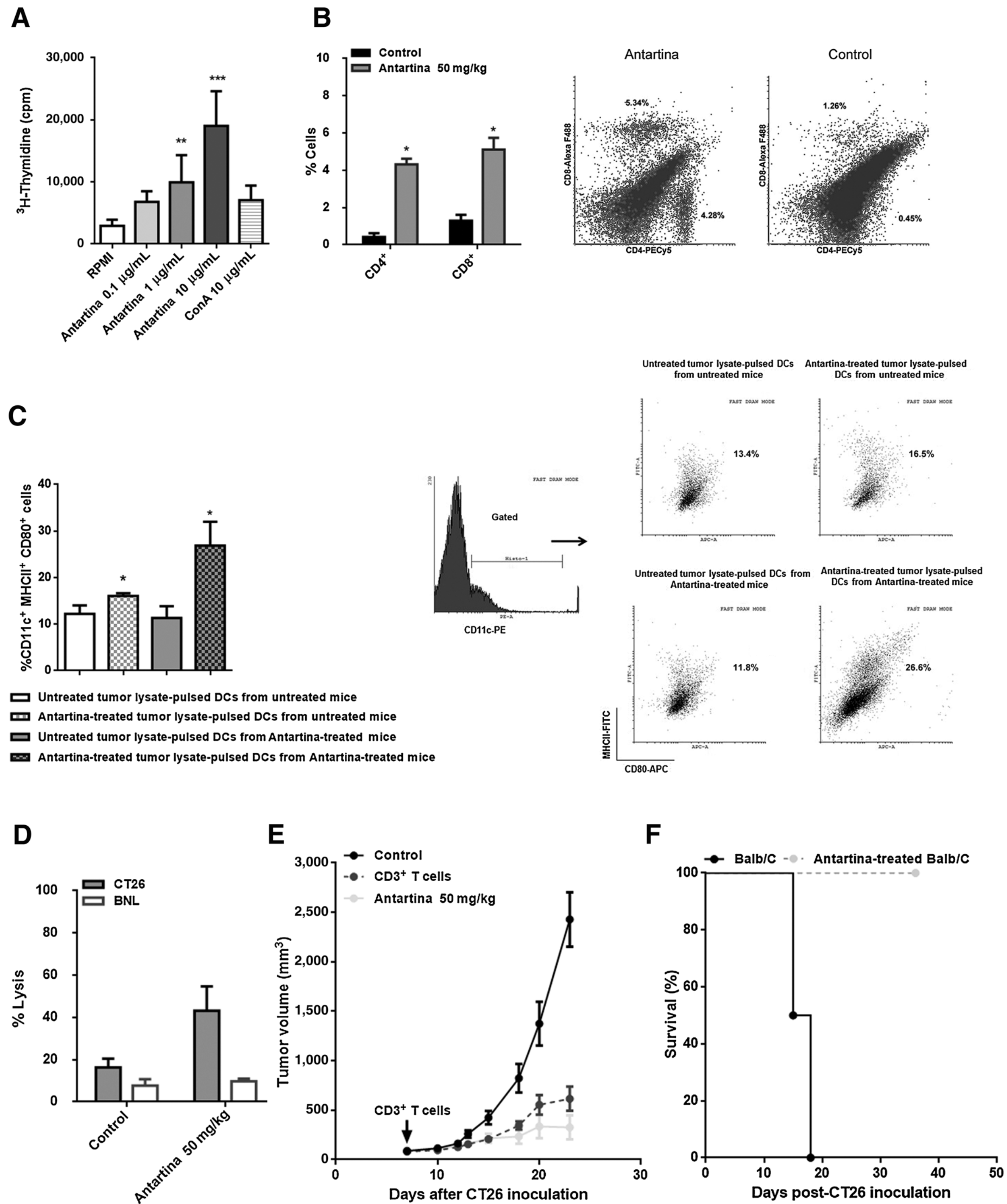

Figure 6.

Antartina therapy generates therapeutic antitumor immunity. **A,** Induction of splenocytes proliferation by Antartina. Splenocytes from naïve mice were exposed to 10 μg/mL Antartina. ^{3}H-Thymidine incorporation (cpm ± SD) are shown. **, $P < 0.01$ RPMI versus 1 μg/mL and ***, $P < 0.001$ RPMI versus 10 μg/mL Antartina Kruskal–Wallis test. **B,** Comparative analysis of CD4$^+$ and CD8$^+$ T cells in tumor tissues from untreated or 50 mg/kg Antartina-treated mice. The frequency of CD8$^+$ cells and CD4$^+$ T cells was significantly higher in tumor tissue after Antartina therapy. *, $P < 0.05$ control versus Antartina on day 18, Mann–Whitney test. **C,** Antartina increases colorectal carcinoma tumor immunogenicity. Tumor lysate from mice treated with Antartina enhances DCs maturation: phenotypic analyses of pulsed DCs stained with anti-CD11c, MHC-II, and CD80. The CD11c$^+$ was gated and the coexpression of several markers was analyzed. *, $P < 0.05$ Kruskal–Wallis test. **D,** Antartina induces a potent specific cytotoxic T-cell response against CT26 cells. Splenocytes derived from Antartina-treated and cured mice exerted a potent CTL activity against CT26 cells. ***, $P < 0.001$ control versus Antartina, Student *t* test. BNL hepatoma cell line was used as specificity control for CT26 cells. **E,** Antitumor effect of adoptively transferred CD3$^+$ T cells on CT26 tumor-bearing mice. Adoptive transfer of specific CD3$^+$ T cells induced a significant inhibition of tumor growth in comparison with controls. Bars represent the average of measures of each group ($n = 6$/group) ± SEM. *, $P < 0.05$, Kruskal–Wallis test. **F,** Antartina induces long-term antitumor immunity against CT26 colorectal carcinoma cells. Naïve BALB/c ($n = 4$) and cured mice ($n = 3$) were rechallenged with 5×10^5 CT26 cells into the left flank. Results were expressed as the percentage of animal survival (Kaplan–Meier, log-rank test, $P < 0.05$).

cell death of cancer cells, significant clinical responses were not observed after their application (39).

The presence of immune cells, in particular $CD8^+$ T cells within the tumor microenvironment, is considered a marker of good prognosis for many tumor types, including colorectal carcinoma (41, 42). In line with this, immunotherapy using immunostimulatory mAbs is gaining much attention in the clinic (43, 44) in particular with the use of checkpoint inhibitors (45). However, the elimination of inhibitory pathways may not be enough to cure cancer, and strategies aimed at increasing the amount of specific tumor-infiltrating immune cells could be similarly important.

We observed a remarkable macroscopic effect of Antartina on colorectal carcinoma tumors, which was histologically supported by the presence of extensive areas of necrosis and intense mononuclear inflammatory infiltrate mainly due to $CD4^+$ and $CD8^+$ T cells. These observations correlate with the potent specific cytotoxic T-cell response observed. Taken together, these data suggest that Antartina might modulate the immune system as well as the tumor microenvironment leading to the inhibition of colorectal carcinoma tumor growth and even to the eradication of near 30% of tumors. Importantly, our molecule generated long-term immune memory against tumor rechallenge in cured mice after Antartina therapy.

The immunomodulatory effect of Antartina on the immune system may be beneficial for the designing of combinatorial antitumor strategies. The ability of Antartina to recruit effector cells into the tumor microenvironment could enhance for instance the activity of immune checkpoint inhibitors (e.g., nivolumab or ipilimumab) in colorectal carcinoma with low tumor inflammatory infiltrate (46, 47).

On the other hand, many cancer chemotherapeutic drugs and some immunostimulatory agents can produce toxicity, even at the therapeutic doses, and toxicity remains one of the most important barriers to the administration of curative doses of the drug. It is clear that not only efficient but also nontoxic therapies are needed. We characterized the toxicology profile of Antartina therapy in mice and found that all animals remained healthy and tolerated repeated doses with no signs of toxicity.

All in all, our study demonstrated that the synthetic molecule Antartina has potent immunostimulatory properties and that it deserves further evaluation as a potential anticancer molecule for patients with advanced colorectal carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Other (characterization of the molecular structure of isolated and synthesized compounds by NMR and mass spectrometry techniques): A. Salgado

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