Les effets anti-tumoraux des inhibiteurs d’HDAC dans un modèle in ovo de cancer pancréatique humain sont significativement améliorés par l’inhibition simultanée de la cyclooxygénase 2.

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VII. ANNEXES
Annexes

Annexe 1

Effect of HDAC Inhibition in a Human Pancreas Cancer Model Is Significantly Improved by the Simultaneous Inhibition of Cyclooxygenase 2.

Peulen, O*. Gonzalez, A*. Peixoto, P. Turtoi, A. Mottet, D. Delvenne, P. and Castronovo, V.

_PlosOne, 2013. 8(9)._  

Annexe 2

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Peulen, O. Gonzalez, A. Peixoto, P. Turtoi, A. Mottet, D. Delvenne, P. and Castronovo, V.

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The Anti-Tumor Effect of HDAC Inhibition in a Human Pancreas Cancer Model Is Significantly Improved by the Simultaneous Inhibition of Cyclooxygenase 2

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Abstract
Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death worldwide, with no satisfactory treatment to date. In this study, we tested whether the combined inhibition of cyclooxygenase-2 (COX-2) and class I histone deacetylase (HDAC) may results in a better control of pancreatic ductal adenocarcinoma. The impact of the concomitant HDAC and COX-2 inhibition on cell growth, apoptosis and cell cycle was assessed first in vitro on human pancreas BxPC-3, PANC-1 or CFPAC-1 cells treated with chemical inhibitors (SAHA, MS-275 and celecoxib) or HDAC1/2/3/7 siRNA. To test the potential antitumoral activity of this combination in vivo, we have developed and characterized, a refined chick chorioallantoic membrane tumor model that histologically and proteomically mimics human pancreatic ductal adenocarcinoma. The combination of HDAC1/3 and COX-2 inhibition significantly impaired proliferation of BxPC-3 cells in vitro and stalled entirely the BxPC-3 cells tumor growth onto the chorioallantoic membrane in vivo. The combination was more effective than either drug used alone. Consistently, we showed that both HDAC1 and HDAC3 inhibition induced the expression of COX-2 via the NF-kB pathway. Our data demonstrate, for the first time in a Pancreatic Ductal Adenocarcinoma (PDAC) model, a significant action of HDAC and COX-2 inhibitors on cancer cell growth, which sets the basis for the development of potentially effective new combinatory therapies for pancreatic ductal adenocarcinoma patients.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) lists among the most deadly form of cancers [1]. Early-stage of the disease is clinically silent and the diagnosis of the disease is mostly made at an advanced stage. This late diagnosis contributes to one of the lowest 5-year survival rate (only 3%) [2]. Today PDAC are treated by surgery and/or adjuvant therapy with gemcitabine, increasing only slightly the median survival of the patients. There is therefore an urgent need to develop new effective therapies for PDAC patients.

There are abundant evidence indicating that deregulation of histone acetylation contributes to pancreas cancer development and progression [3]. Histone deacetylases (HDAC) represent a family of enzymes that regulate paramount cellular activities including epigenetic silencing of tumor suppressor genes and modulation of protein functions. We and others have shown that HDAC inhibition exerts both anti-cancer and anti-angiogenesis activities [4–6]. HDAC expression is altered in PDAC, including HDAC1, HDAC2, HDAC3 and HDAC7 [7–10]. Preclinical studies have suggested that HDAC inhibition hold significant potential for the development of new anticancer therapies [11]. Accordingly, several HDAC inhibitors have been recently approved by the Food and Drug Administration for the treatment of Cutaneous T-Cell Lymphoma while new molecules are currently in phase III clinical trials. However, when used in monotherapy, HDAC inhibitors showed limited efficacy in various solid malignancies, including PDAC [3,12,13]. Indeed, LAQ824 or MS-275 have been evaluated in phase I clinical trials in solid cancers, including PDAC, without any objective clinical response [14,15]. Alternatively, HDAC inhibitors have been used in combined therapy strategies [16,17], with some combinations generating promising effects for human PDAC in vitro [18–21] or in experimental tumors [22]. Unfortunately, these results do not translate in clinical trials [23,24].

The lack of efficacy of HDAC inhibitors in pancreatic cancer could be linked to the pleiotropic activities of HDACs in cell biology [25,26] leading to undesired pro-cancer effects. For example, a recent study demonstrated that pan-HDAC inhibitors induce cyclooxygenase-2 (COX-2) expression in lung cancer cells, leading to a stimulation of endothelial cell proliferation [27]. Since
COX-2 has been also associated to pancreatic cancer cell proliferation [28] or tumor growth [29–31], we hypothesized that COX-2 overexpression may also be induced in PDAC when treated with HDAC inhibitors, leading to reduced efficiency and hence therapeutic failure.

To test the biological relevance of combining class I HDAC and COX-2 inhibitors in vivo, we devised a refined PDAC chick chorioallantoic membrane (CAM) model based on our previous work [32]. The CAM model has been successfully used with several cell lines to produce tumors [33,34]. Similarly to the murine model, most steps of tumor progression are recapitulated in a very short period of time [35]. Previously, BxPC-3 pancreatic cancer cells were already demonstrated to produce vascularized 100 µm long tumor nodes on CAM [32]. However, the small size of the nodules represented a significant limitation for structural observation, accurate volume evaluation and study of drug efficacy. Here, we have established and implemented a refined BxPC-3 PDAC model featuring a dramatic increase (64-fold) in tumor size and displaying structural architecture and protein expression mimicking human PDAC. This model was successfully exploited to demonstrate that the combination of class I HDAC and COX-2 inhibitors result in a complete tumor growth inhibition.

**Materials and Methods**

**Cells and chemicals**

BxPC-3 (ATCC CRL-1687), PANC-1 (ATCC CRL-1469) and CFPAC-1 (ATCC CRL-1918) are human pancreatic cancer cell lines derived respectively from PDAC [36], pancreas duct epithelioid carcinoma [37] and PDAC liver metastasis [38]. BxPC-3 were a generous gift from Prof. Bikfalvi (Inserm u1029, Bordeaux, France), Panc-1 were a generous gift from Prof. Muller and Burtea (NMR Laboratory, University of Mons, Belgium). CFPAC-1 were bought from ATCC. Celecoxib was obtained from the University Pharmacy (Kemprotec Ltd, Middlesbrough, UK). MS-275 and SAHA were purchased from Enoz Life Sciences (Antwerpen, Belgium). Other chemicals were purchased from Sigma (Bornem, Belgium).

**Cell culture**

BxPC-3 human pancreatic cancer cell line were maintained in RPMI1640 medium supplemented with glucose (2.5 g/L), sodium pyruvate (1 mM) and FBS (10%). PANC-1 were maintained in DMEM supplemented with FBS (10%). CFPAC-1 were maintained in Iscove’s Modified Dulbecco’s Medium with FBS (10%). Cells were treated with MS-275, celecoxib or combination of both as well as with suberoylanilide hydroxamic acid (SAHA) solubilized in medium with 0.1% DMSO.

**Small interfering RNA transfection**

HDAC-specific small interfering RNA (siRNA) were synthesized by Eurogentec (Seraing, Belgium). NF-kB p65 SMARTpool siRNA were bought from Thermo Fisher-Dharmacon (Whaltham, MA). Lipopectamine-mediated transfections were performed at a siRNA concentration of 40 nM following manufacturer’s recommendations (Life Technologies, Carlsbad, NM). GL3 was an irrelevant siRNA targeting luciferase. siRNA sequences were published previously [5].

**Cell growth**

Equal densities of cells were seeded in complete medium and were harvested at the indicated time-points. The cell numbers were indirectly determined using Hoechst incorporation. Results were expressed as DNA content.

**Western-blotting**

BxPC-3 cells or frozen tumors were disrupted in lysis buffer (1% SDS, 40 mM Tris-HCl pH7.5) in the presence of protease and phosphatase inhibitors. Proteins were separated by SDS-PAGE (6–12.5%) then electrotransferred on nitrocellulose membranes. Following primary antibodies were used: anti-COX-2 (Cayman Chemicals, Ann Arbor, MI), anti-HDAC1 (Cell Signalling, Danvers, MA), anti-HDAC2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HDAC3 (Cell Signalling, Danvers, MA), anti-acetylated-Histone-3 (Millipore, Billerica, MA), anti-HDAC7 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-IkBz (Cell Signalling, Danvers, MA), anti-p65 (Cell signalling, Danvers, MA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 (BD Biosciences, Franklin Lakes, NJ), anti-pRB (BD Biosciences, Franklin Lakes, NJ), anti-E2F1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MEK2 (Cell signalling, Danvers, MA), anti-ORC2 (Cell signalling, Danvers, MA), anti-caspase-3 (Cell Signalling, Danvers, MA) and anti-HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed using appropriate secondary antibody conjugated with horseradish peroxidase.

**Quantitative real-time RT-PCR**

Total RNA extraction and quantitative real-time RT-PCR were performed as previously described [39]. Human COX-2 expression was detected using a commercial RT-qPCR TaqMan assay (Hs00153133-m1; Applied Biosystems, Carlsbad, NM). Human IL-8 expression was detected using specific forward (5′-GAAGGAACCATCTCTACTGTGTGTAA-3′) and reverse (5′-ATCGAGGAAGGCTGCCAAAGAG-3′) primers synthesized by Eurogentec (Seraing, Belgium).

**Annexin V/propiodium iodide staining**

Apoptotic cells were determined by annexin V-FTTC and non-vital dye propidium iodide (PI) staining with a FITC-Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s instructions. Flow cytometry was performed on a FACSCalibur II™ and samples were analyzed using CellQuest™ software (BD Biosciences, Franklin Lakes, NJ).

**Cell cycle analysis**

The relative percentage of cells in each stage of the cell cycle was analyzed as previously described [33] by flow cytometric analysis with FACSCalibur II™ and ModFit LT™ program.

**Tumor growth on CAM**

Fertilized chicken eggs were opened as previously described [32]. On post-fertilization day 11, CAM surface was gently scratched with a needle and 3.5×10^6 BxPC-3, PANC-1 or CFPAC-1 cells in suspension with 50% matrigel in a final volume of 100 µL were grafted on the CAM enclosed by a 6-mm plastic ring. The implantation day was considered as day 0 of tumor development. Drugs (celecoxib 8 µM and/or MS-275 0.2 µM in a 30 µL final volume) were applied daily directly on tumor starting at day 2. At day 7, the tumors were excised from the CAM and digital pictures were taken using a stereomicroscope. Tumor volume was calculated using an ellipsoid formula: Volume = (4/3)π(Z_1×Z_2×Z_3)/3 where Z_1–Z_3 are the main radius of the tumor.
Ethics statement

All animal experiments were approved by the Animal Welfare Committee of the University of Li`ge (approval #1278).

Histology procedure

BxPC-3 tumors were washed in PBS and then fixed in 4% paraformaldehyde for 30min at 4°C. The tumors were embedded in paraffin and 5 µm sections were stained with Hematoxylin-eosin or Masson’s trichrome.

Immunoperoxidase and amylase-periodic acid Schiff (PAS) staining were performed on 5 µm sections, respectively, with the BenchMark XT IHC/ISH automated stainer and the NexES Special Stains (Ventana Medical Systems Inc, Tucson, AZ) according to the manufacturer’s instructions. Following antibodies were used: anti-cytokeratin 7 (CK7 - Dako, Glostrup, Denmark), anti-cytokeratin 19 (CK19 - Roche Diagnostics, Vilvoorde, Belgium), anti-cytokeratin 20 (CK20 - Dako, Glostrup, Denmark), anti-CD56 (Novocastra, Leica Microsystems Inc, Buffalo Grove, IL), anti-carcinoembryonic antigen (CEA - Roche Diagnostics, Vilvoorde, Belgium), anti-Ki67 (Dako, Glostrup, Denmark), anti-latent transforming growth factor-beta binding protein 2 (LTBP2 – Santa Cruz Biotechnology, Santa Cruz, CA), anti-transforming growth factor beta-induced (TGFB1 - Cell Signalling, Danvers, MA), anti-myosin (Sigma, Bornem, Belgium) and anti-desmin (Dako, Glostrup, Denmark) were used for the primary reaction.

Ki67 quantification was performed on randomly taken pictures (3 pictures from each tumor, 3 tumors in each experimental group). After channel splitting, blue channel pictures were binarized according to the brightness. The size of the area occupied by all cells or by Ki67-positive cells was measured using imageJ 1.46r software.

In order to visualize the tumor vasculature, thick rehydrated tissue sections (35 µm) were incubated for 30min in the dark with 0.05% Triton X-100 in PBS containing 5 µg/mL Sambucus nigra agglutinin (SNA, Vector Laboratories, Burlingame, CA). The sections were washed with 0.05% Triton X-100 in PBS and visualized with confocal microscope (Leica SP2). Three-dimensional images were reconstructed with Imaris software (Bitplane Scientific Software, Zurich, Switzerland).

Statistical analysis

All results were reported as means with standard deviation. Statistical analysis was performed using one-way or two-way ANOVA depending on the number of grouping factors. Group

Figure 1. Effect of HDAC silencing or inhibition on BxPC-3 cell proliferation. (A) Time-dependent and dose-dependent effects of SAHA on cell proliferation. (B) Time-dependent effect of class IIa HDAC7 silencing on cell proliferation. HDAC7 expression was detected by western-blot 48h after siRNA transfection. HSC70 was used as a loading control. (C) Time-dependent effect of class I HDAC1 or –3 silencing on cell proliferation. HDAC1 and HDAC3 expression was detected by western-blot 48h after siRNA transfection. HSC70 was used as a loading control. (D) Time-dependent and dose-dependent effects of MS-275 on cell proliferation ***P < .001 versus DMSO or GL3 conditions. Results are expressed as mean ± s.d., n≥3 in each condition.

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means were compared by a Bonferroni’s post-test. \( P < .05 \) was considered as statistically significant. All experiments were performed as 3 independent biological replicates.

**Results**

Class I HDAC inhibition reduced pancreas cancer cell growth in vitro

BxPC-3 cells have been described to express altered levels of class I HDAC1, HDAC3 and class II HDAC7 [40,41]. To evaluate the role of these HDAC in BxPC-3 cells, we first examined their time-dependent and concentration-dependent growth in presence of SAHA, a class I/II inhibitor (Figure 1A). Our results confirmed that BxPC-3 cells were sensitive to SAHA, with a 50% growth reduction (\( P < .001 \)) observed at 5 \( \mu \text{M} \). Next, we selectively silenced HDAC1, –3 or –7 using siRNA to examine the individual involvement of these HDAC in the SAHA-induced growth reduction. HDAC7 silencing did not affect cell growth (Figure 1B). However, HDAC1 and HDAC3 silencing reduced significantly BxPC-3 cell growth by respectively 50% (\( P < .001 \)) and 20% (\( P < .001 \)) (Figure 1C). In order to evaluate this decrease in cell growth with clinically compatible drug, we evaluated the time-dependent and concentration-dependent growth of BxPC-3 cells in presence of MS-275 (HDAC1 and HDAC3 inhibitor). MS-275 (1 \( \mu \text{M} \)) reduced BxPC-3 cell growth by 50% (\( P < .001 \)) whereas 5 \( \mu \text{M} \) abolished completely the growth (\( P < .001 \)) (Figure 1D).

Class I HDAC inhibition induced COX-2 expression in vitro

The limited efficiency of HDAC inhibitors in clinical trials including PDAC patients could be explained, at least in part, by the potential up regulation of the expression of COX-2 in pancreatic malignant cells. To evaluate this hypothesis, we first analyzed COX-2 expression in BxPC-3 cells silenced for HDAC1, HDAC2, HDAC3 or treated with MS-275. HDAC1 or HDAC3 repression induced respectively a 6.3-fold and a 4.8-fold increase of COX-2 expression at protein level (Figure 2A) while HDAC2 repression induced an HDAC2 overexpression.
Treatment of BxPC-3 cells with MS-275 showed similar effects on COX-2 accumulation in a concentration-depend manner (Figure 2C). To determine whether COX-2 induction occurs at transcriptional level, we analyzed COX-2 mRNA level by RT-qPCR following 6, 12, and 24h of MS-275 treatment. We found that COX-2 gene expression was up-regulated following the MS-275 treatment in a time-dependent manner (Figure 2D).

To study the mechanisms by which class I HDAC inhibition induces COX-2, we explored the known link between NF-kB and HDAC1/3 [42,43] and tested the possibility that MS-275-induced COX-2 expression could be NF-kB dependent. Accordingly, we co-treated cells with MS-275 and BAY-11-7082, an IkBα kinase (IKK) inhibitor. BAY-11-7082 reduced by 30% to 90% the COX-2 expression following respectively 6h to 48h of MS-275 treatment (Figure 3A), suggesting the MS-275-induced expression of COX-2 is, at least in part, NF-kB dependent. This hypothesis was supported by p65-silencing and p65 translocation to the nucleus. COX-2 expression was induced by a 24h treatment with MS-275 and was prevented by p65 siRNA (Figure 3B). Moreover, 24h MS-275 treatment induced an increase by 50% of the p65 protein level in the cytoplasm and in the chromatin fraction of BxPC-3 cells (Figure 3C). The same MS-275 treatment induced the gene expression of IL-8 (Figure 3D), a direct target of NF-kB.

Combined inhibition of class I HDAC and COX-2 inhibits cell growth in vitro

In order to validate our hypothesis that class I HDAC inhibition mediated induction of COX-2 might contribute to the low efficiency of HDAC based therapy in PDAC patients, we have combined the latter with celecoxib, a selective COX-2 inhibitor at IC50 (respectively 1 μM of MS-275 and 10 μM of celecoxib). The MS-275-induced COX-2 overexpression led to a 50% increase of PGE2 concentration in the culture media (Figure 4A). BxPC-3 cell treatment with celecoxib alone or in combination with MS-275 reduced significantly the PGE2 concentration in the cell media. We then asked the question whether this reduction is due to induction of apoptosis and performed an annexin V/propidium iodide staining at 24, 48 and 72h (Figure 4C) following the treatment. None of the individual drugs nor their combination were able to induce apoptosis. These results were

Figure 3. Effect of HDAC inhibition on NF-kB activation in BxPC-3 cells. (A) Effect of an IKK inhibitor (10 μM BAY-11-7082) on 1 μM MS-275-induced COX-2 expression. Phospho-IkBα was used as a control of BAY-11-7082 treatment efficacy. HSC70 was used as a loading control. Densitometry was expressed as a COX-2/HSC70 or IkBα/HSC70 ratio. (B) Western-blot detection of COX-2 in 20 μg BxPC-3 proteins after 1 μM MS-275 treatment and p65 siRNA transfection. HSC70 was used as a loading control. (C) Western-blot detection of p65 in 15 μg BxPC-3 cytoplasm, nucleoplasm or chromatin-associated proteins after 1 μM MS-275 treatment. MEK2 and ORC2 were used as a loading control respectively in cytoplasm and chromatin fractions. Densitometry was expressed as a p65/MEK2 or p65/ORC2 ratio. (D) Time-dependent relative expression of IL-8 mRNA in BxPC-3 cells treated with 1 μM MS-275, 10 μM Celecoxib or a combination of the drugs. Results are expressed as mean ± s.d. ***P<.001, *P<.05 versus DMSO. n≥3 in each condition.

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confirmed by western-blot, showing intact caspase-3 in all samples (Figure 4C). To further investigate the mechanisms of the observed cell growth arrest, we next examined the effect of MS-275/celecoxib combination on the cell cycle (Figure 4D). MS-275 alone, but not celecoxib, increased the proportion of cell in G1 by 50% at 48h. However, MS-275/celecoxib combination decreased significantly (P < .001) the proportion of cells in S phase at 24 (-74%), 48 (-92%) and 72h (-82%) and increased significantly (P < .001) the proportion in G1 phase at 24 (+48%), 48 (+119%) and 72h (+80%). To validate these results we analyzed by western blot the expression of cell cycle markers and found a clear accumulation of p21WAF1 and p27Kip1, two cell cycle inhibitors, at 24h and 48h after the co-administration of MS-275 and celecoxib (Figure 4E). Consistently, the hyperphosphorylated form of pRb was less abundant when BxPC-3 cells were co-treated with MS-275/celecoxib. The hypophosphorylated form of pRb appeared with the co-inhibition of class I HDAC and COX-2. The whole pRb protein disappeared at 48h after the cotreatment. This disappearance was already observed by others after a p21WAF1 or p27Kip1 accumulation [44]. The E2F1 transcription factor, a S-phase orchestrator, became undetectable 48h after co-administration of MS-275 and celecoxib. These results show that cellular growth inhibition is associated to a G0/G1 phase blockage.

BxPC-3 is a PDAC cell line characterized by its KRAS wildtype, while mutations of the gene coding for this protein is the most common genetic alteration observed in human PDAC. However, BxPC-3 cells overexpress COX-2, a situation noted in 50% of human PDAC. We have decided to extend our observations regarding the interest of the combined treatment in pancreatic cancer by examining the efficiency of such combined treatment on two human pancreas cell lines with reported KRAS mutations. The first cell line was PANC-1 ([12 ASP]-KRAS) in which COX-2 was undetected at the protein level [45]. The second cell line was CFPAC-1 ([12 VAL]-KRAS) but in which COX-2 was detected at protein level [45].

PANC-1 cell line was cultured with MS-275, celecoxib or both drugs in combination. Celecoxib 10 μM did not alter cell growth when MS-275 1 μM reduced significantly (p < .001) cell growth by 32%. The combination of the two drugs reduced the PANC-1 cell growth (49%, P < .001). Here, the combination-induced growth inhibition was not significantly different from the MS-275-induced one (Figure 5A). In this cell line, MS-275 did not induce the expression of COX-2 (data not shown).

CFPAC-1 cell line was cultured in the same conditions. Celecoxib 10 μM reduced cell growth by 54% (p < .001) and MS-275 1 μM reduced cell growth by 59% (p < .001). Here, the
Combination of the two drugs reduced significantly (79%, P<0.001) CFPAC-1 cell growth in comparison to either drug alone (Figure 5B). We then analyzed by western blot the expression of COX-2 and cell cycle markers in CFPAC-1 cells 48h after drugs administration. We showed an MS-275-induced accumulation of COX-2 like in BxPC-3 cells (Figure 5C). We found also an accumulation of p21WAF1 and p27Kip1 after the co-administration of MS-275 and celecoxib (Figure 5C), suggesting a cell cycle arrest.

BxPC-3 CAM tumor mimics human PDAC

The evaluation of new drugs or drug combinations for pancreas cancer will be eased by the availability of easy, ethically and economically sustainable animal models. Thus, we have undertaken to refine a human pancreas chorioallantoic membrane (CAM) model based on our initial work [32]. Embedding BxPC-3 cells into matrigel prior to CAM implantation generated a major improvement in the tumor volume. Indeed, following implantation, the tumor volume increased linearly (r² = 0.87) until day 7 (Figure 6A). At the time of tumor collection (day 7), an average tumor volume of 59.95 ± 15.34 mm³ (n = 10) was observed. BxPC-3 CAM tumors grew inside the CAM connective tissue as a unique spheric nodule. The same procedure was followed for BxPC-3, PANC-1 and CFPAC-1 cell lines. PANC-1 did not grow on CAM when CFPAC-1 grew as very small nodules (1 mm long).

BxPC-3 CAM tumor histology (Figure 6B) revealed large islets of cohesive cells, some of which showed a nascent central lumen and were isolated from each other by a collagen-containing extracellular matrix with several sparse fibroblast-like cells demonstrating the presence of an interstitial stroma.

To further validate our human pancreas cancer CAM model, we compared the expression of the cytokeratin-7, -19, -20, CD56, CEA and Ki67 using immunohistochemistry to human PDAC. We also checked for mucin and proteoglycan production utilizing the PAS staining. Tumoral cells from both BxPC-3 CAM tumor and PDAC samples were strongly positive for cytokeratin-7 and -19, CEA and Ki67 (Figure 6C) but negative for cytokeratin-20 and CD56 (data not shown). Both tumors were positive for PAS staining. Altogether, the data showed remarkable histology and biomarker expression similarities between the BxPC-3 CAM model and PDAC from human patients.

Furthermore, our recent work on targetable biomarkers in human PDAC [46] identified several biomarker candidates among which myoferlin, transforming growth factor beta-induced and latent-transforming growth factor beta-binding protein 2. Immunohistochemistry and western-blot confirmed the presence of these new PDAC biomarkers in the BxPC-3 CAM tumors (Figure 7A–B). Finally, using western blot we confirmed that HDAC1, HDAC2, HDAC3 and COX-2 are expressed in the BxPC-3 CAM tumor (Figure 7A).

We next demonstrated that tumors were functionally vascularized. BxPC-3 CAM blood vessels were stained by FITC-conjugated SNA and 3D reconstructed after confocal acquisition. BxPC-3 CAM tumors displayed blood vessels around pancreatic islets (Figure 8A). The fluorescence of tumor stroma after
fluorescent dye injection in the CAM vasculature confirms that the vessels are functional (Figure 8B) and the detection of desmin positive pericytes suggests vessel stabilization (Figure 8C).

Next, BxPC-3 tumors were treated beginning day 2 either with 8 μM celecoxib or 0.2 μM MS-275 or with a combination of two drugs at their respective concentrations. MS-275 concentration was chosen to fit with the plasmatic concentration measured in Human in a 5 mg/m2 weekly dosing schedule [15]. While celecoxib alone did not affect tumor growth, MS-275 alone induced a decreased of tumor growth by 50% (P<.001) and induced the expression of COX-2. Combination of celecoxib and MS-275 completely abolished (P<.001) tumor growth, leading to no change in tumor volume compared to the beginning of treatment (Figure 9A-B). Tumors treated with MS-275 overexpressed COX-2 (Figure 9C). Tumors treated with combination of celecoxib and MS-275 revealed empty spaces inside the tumor (Figure 9D). We then asked whether this reduction of tumor volume is due to induction of apoptosis or to proliferation arrest. Tumors treated with MS-275, celecoxib or both drugs were submitted to a cleaved caspase-3 detection and were labeled for Ki67. The full-length caspase-3 was detected in all samples but no cleaved caspase-3 was observed (Figure 9E). The relative Ki67-positive area was slightly but significantly reduced by the combination of HDAC and COX-2 inhibitors (Figure 9F).

Discussion
The potential interest of anti-HDAC treatment strategies for PDAC is supported by several preclinical studies [18,19,22,47–50]. In agreement with these studies, we showed that pan-HDAC inhibitor SAHA was able to reduce significantly pancreatic cancer cell growth. Following the rationale that HDAC7, HDAC3 and HDAC1 have been reported to be over-expressed in the PDAC [8–10] we have examined their individual roles with respect to their ability to control BxPC-3 cell growth. The results demonstrated that HDAC7 silencing was unable to decrease the cell growth while HDAC1 and HDAC3 inhibition or silencing reduced significantly the BxPC-3 cell growth highlighting the importance of these enzymes in PDAC patients. However, the results of clinical studies where HDAC inhibitors are used show only limited or no ability to affect tumor development [3,13]. This is likely to be related to the pleiotropic activities of HDAC including some that might promote tumor progression. In this line, HDAC1, –2 and –3 may have been shown to regulate the function of RelA/p65 subunits of NF-kB. Class I HDAC1 can indeed interact with RelA/p65 acting as a corepressor to negatively regulate NF-kB activity.
regulate its transcriptional activity [43]. HDAC3-mediated deacetylation of RelA/p65 promotes its binding to IKBα leading to cytosolic sequestration [42] and NF-kB repression. In parallel, HDAC2 was also overexpressed in PDAC and was shown to regulate NF-kB activity without direct interaction with p65 [43]. As a consequence, class I HDAC inhibition could induce the transcriptional activation of NF-kB-driven genes. Consistently, a significant COX-2 induction was recently showed in lung cancer cells following trichostatin A or SAHA treatment [27]. Here, we showed, for the first time, that the class I HDAC chemical inhibitor MS-275 and selective silencing of both HDAC1 and HDAC3 are able to induce the transcription of COX-2 gene and the accumulation of the functional enzyme independently of the KRAS status. Conversely, HDAC2 silencing does not elicit COX-2 accumulation but reduce its expression. COX-2 is considered to be part of the positive feedback loop amplifying Ras activity to a pathological level causing inflammation and cancer [51]. Moreover, COX-2 was demonstrated to confer a growth advantage to pancreatic cancer cells [52]. These results together with our findings suggest the potential interest in inhibiting COX-2 activity while subjecting COX-2 positive (about 50-60% of the cases [53]) PDAC patients to anti-HDAC treatments. This can be easily achieved because several molecules, including the celecoxib [54], were developed in order to inhibit specifically COX-2. Celecoxib was found to significantly decrease or delay pancreatic cancer progression in animal model [29,55]. Keeping these findings in mind, we combined class I HDAC and COX-2 inhibitors and test their efficiency to control tumor growth. The co-treatment reduced the pancreas cancer cell growth by blocking cells in G0/G1 state. This is probably a mechanism that could explain the effects observed in vivo, where the combination of two drugs completely stalled the tumor growth. Importantly, the inhibition of tumor growth was observed with drug concentrations 10-fold lower than the concentrations needed if the drugs were used individually [56,57]. This represents a considerable advantage for a putative clinical use regarding the possible undesired effects. However, the in vivo model used in this work remains very simple compared to the complexity of the pathology in human. Moreover, the cell line used to grow the tumor in ovo is a limitation as it does not harbor constitutively active Kras which is the most common genetic alteration in human PDAC. In consequence, in vivo studies in genetically-engineered mouse models of PDAC are more than necessary before entering potential clinical trials with combined treatment, especially in the case of patients harboring KRAS mutation. Several models are now available to recapitulate the disease [58].

One additional outcome of the current study is the development and characterization of a refined animal model of PDAC recapitulating all the main features observed in human tumors. We have based our development on a model we previously set-up [32] but which did not provide with the possibility to efficiently test experimental therapies. Following extensive method development
we have established means to produce larger tumors, bearing fully functional blood vessels. The clinical relevance of this improved model is supported by the CK7+/CK19+/CEA+/Ki67+/CD56+ immunodetection. CK7 and CK20 expression has been shown to be useful in the differential diagnosis of several carcinomas of epithelial origin. According to Lee et al. [59] 95% of PDAC are CK7+, 100% are CK19+ and 73% are CK20+. In pancreas carcinomas the proportion of cells stained for CEA and the Ki-67 index were respectively increased 3-fold and 10-fold in comparison with the normal tissue [60,61]. CD56 staining was found negative in all cases of human PDAC [62]. These biomarkers, together with the presence of mucin are the main hallmarks of PDAC [63].

Recently, we have discovered several biomarkers of human PDAC that bear therapeutic potential [46]. These antigens were also present in our CAM tumor model, supporting its similarity with human cancer and providing the research community with a rapid and cost effective model for pancreas cancer research such as our present demonstration of the benefit to combine COX-2 and HDAC inhibition for optimal anti tumor activity.

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Author Contributions

Conceived and designed the experiments: OP VC. Performed the experiments: AG PP PD. Analyzed the data: OP AG DM AT VC. Contributed reagents/materials/analysis tools: PD. Wrote the paper: OP VC. Obtained the permission to use the PANC-1 cell line: OP.
References


HDAC5 is required for maintenance of pericentric heterochromatin, and controls cell-cycle progression and survival of human cancer cells.

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Histone deacetylases (HDACs) form a family of enzymes, which have fundamental roles in the epigenetic regulation of gene expression and contribute to the growth, differentiation, and apoptosis of cancer cells. In this study, we further investigated the biological function of HDAC5 in cancer cells. We found HDAC5 is associated with actively replicating pericentric heterochromatin during late S phase. We demonstrated that specific depletion of HDAC5 by RNA interference resulted in profound changes in the heterochromatin structure and slowed down ongoing replication forks. This defect in heterochromatin maintenance and assembly are sensed by DNA damage checkpoint pathways, which triggered cancer cells to autophagy and apoptosis, and arrested their growth both in vitro and in vivo. Finally, we also demonstrated that HDAC5 depletion led to enhanced sensitivity of DNA to DNA-damaging agents, suggesting that heterochromatin-decondensation induced by histone HDAC5 silencing may enhance the efficacy of cytotoxic agents that act by targeting DNA in vitro. Together, these results highlighted for the first time an unrecognized link between HDAC5 and the maintenance/assembly of heterochromatin structure, and demonstrated that its specific inhibition might contribute to increase the efficacy of DNA alteration-based cancer therapies in clinic.

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Histone deacetylases (HDACs) are enzymes that modulate the acetylation level of histones and non-histone proteins to regulate gene expression and chromatin structure. Eighteen human HDACs are divided into four classes: class I (HDAC1, 2, 3, 8); class II (HDAC4, 5, 6, 7, 9, 10), subdivided into class IIa (HDAC4, 5, 7) and class IIb (HDAC6 and 10); class III, also called sirtuin proteins (SIRT1–7); and class IV (HDAC11). Several compounds were identified as broad-spectrum inhibitors of classes-I and -II HDAC (HDACi). These HDACIs can cause cell-cycle arrest, activation of programmed cell death (apoptosis/autophagy), or inhibition of angiogenesis. Based on their potent anticancer effects in vitro, several HDACIs are currently being investigated in clinical trials in cancer patients, both as single agents and in combination with other drugs. The FDA (Food and Drug Administration) approval of SAHA (suberoylanilide hydroxamic acid; Zolinza) for treatment of cutaneous T-cell lymphoma validates the concept of HDAC inhibition to treat cancer.

Generally, HDACIs are well tolerated when compared with most of the currently used antitumor treatments. However, some side effects have been reported. So, by targeting the most relevant HDAC members, it may be possible to improve efficacy by removing undesirable toxicities. Preclinical investigations by targeted knockdown of individual HDAC members demonstrated the roles of class IIa HDACs in tumorigenesis. Indeed, we and others have demonstrated that silencing of HDAC4 inhibited cancer cell proliferation in vitro and arrested tumor growth in vivo through epigenetic regulation of p21WAF1/Cip1 gene expression. Recently, Zhu et al. also demonstrated HDAC7 is a crucial player in...
cancer cell proliferation. Together, these findings would suggest that inhibition of class IIa HDACs might be a sufficient strategy to treat cancer. However, the contribution of HDAC5 to tumor progression is largely ignored and needs to be further characterized to determine whether class IIa HDAC members are the most relevant targets in cancer therapy.

The human HDAC5 gene is located on chromosome 17q21, a region which is characterized by losses of chromosomal material in different cancers. Moreover, HDAC5 expression is frequently reduced in cancer such as colon cancer and acute myeloid leukemia, and is associated with poor clinical outcome of lung cancer patients. In contrary, an upregulation of HDAC5 has been observed in high-risk medulloblastoma and its expression is associated with poor survival. Like for many HDACs, HDAC5 is then aberrantly expressed in tumors, suggesting that this HDAC may have a role in tumor progression.

Here, we investigated the function of HDAC5 in cancer cells. We found that its sub-nuclear localization changed during S phase progression, with HDAC5 colocalizing with actively replicating heterochromatic regions during late S phase. We demonstrated that its specific depletion by RNA interference (RNAi) induced a defect in pericentric heterochromatin assembly and slowed down an ongoing replication fork, which consequently induced DNA-damage checkpoint pathways, which leads to cell-cycle blocking, inhibition of cell proliferation, induction of apoptosis as well as autophagy, and, consequently, decreased tumor growth in vivo. Altogether, these findings implicate HDAC5 in the maintenance/assembly of pericentric heterochromatin structure and demonstrate that class IIa HDAC5 can represent a potential target for anticancer therapies.

Results

HDAC5 localizes to pericentromeric heterochromatin primarily during late S phase. To explore the function of HDAC5, we first examined its localization in HeLa cells. Confocal microscopy showed that approximately 85% of asynchronous cells showed a nuclear localization, with more intense foci around the nucleolus (Figure 1A). This localization was observed in different cell types, including MCF-7, MDA-MB-231, endothelial cells, and fibroblasts (data not shown). Transfection with two different efficient HDAC5 small interfering RNA (siRNA) correlated with loss of nuclear HDAC5 foci, excluding a non-specific staining (Supplementary Figures S1A, S1B–S2). This localization was also confirmed with a second anti-HDAC5 antibody (Supplementary Figure S1C). To further characterize the intra-nuclear localization of HDAC5, we performed electron microscopy. Endogenous HDAC5 was detected as individual foci in the nucleus and the clusters showed a preferential colocalization of HDAC5 with pericentric (pericentromeric) heterochromatin (Figure 1B and Supplementary Figure S3). As fluorescence-activated cell sorting (FACS) analysis (see Figure 2a) revealed that an asynchronous population of HeLa cells is composed of 65–70% of cells in G1, 20–25% of cells in S, and 5–10% of cells in G2/M phase, we hypothesized that HDAC5 could target pericentric heterochromatin during different phases of the cell cycle. First, we monitored the localization and expression of HDAC5 during S phase. In early S phase, HDAC5 shows a diffuse nuclear staining, but in late S phase, the patterns were strikingly different, with HDAC5 now colocalizing with proliferating cell nuclear antigen (PCNA) to punctuate foci that are characteristic of late-replicating pericentric heterochromatin (Figure 1C). A colocalization between HDAC5 and heterochromatin protein 1 (HP-1), a heterochromatin marker, in late S phase confirmed that HDAC5 is localized to heterochromatic regions (Figure 1D). The re-entry into S phase was monitored by both FACS (see Figure 1C) and western blotting against cyclin E and phospho-histone H3 on Ser10, two markers of S phase progression, a period during which the global level of HDAC5 did not change (Figure 1E). During mitosis, HDAC5 was not detectable with mitotic chromosome. However, we observed that HDAC5 also associated with heterochromatin in the G1 phase (Supplementary Figure S4). Bearing in mind the importance of HDAC for chromatin condensation, we assessed the impact of HDAC5 depletion on the organization of pericentric heterochromatin by electron microscopy. Electron micrographs of GL3 siRNA/mock-transfected cells revealed dense nucleoli and a condensed pattern of heterochromatin. By contrast, HDAC5-depleted cells showed a reduced number of dense heterochromatin clusters at the periphery of the nucleolus, demonstrating failure of appropriate assembly/maintenance of chromatin structure at pericentric heterochromatin (Figure 1F). To determine whether HDAC5 depletion exerted a more global influence on chromatin organization, we performed a MNaseI (micrococcal nuclease 1) assay. No defects in wrapping of DNA by the histone octamer were observed in the absence of HDAC5 (Figure 1G), suggesting that HDAC5 did not have a role in the assembly/maintenance of nucleosome organization.

HDAC5 depletion affects DNA replication efficiency and cell-cycle progression. Because heterochromatin assembly and DNA replication are tightly coupled, we examined the consequences of HDAC5 depletion on DNA replication and S phase progression. To identify the effect of HDAC5 depletion on global S phase, asynchronous cells were transfected with an HDAC5 siRNA for 24, 48, and 72 h, and then pulsed with 5-bromo-2′-deoxyuridine (BrdU) before FACS analysis. After 24 h, the number of replicating cells in HDAC5-depleted cells was 28.8% lower compared with mock-transfected cells, and most of the cells were blocked in the G1 phase (Figures 2a and b). Forty-eight hours after transfection, the percentage of replicating cells was significantly higher in HDAC5-depleted cells (28.23%) compared with control conditions (Figures 2c and d). After 72 h, no significant changes were observed (Figures 2e and f). These data show that HDAC5 siRNA-transfected cells are first blocked in G1/S and then re-enter S phase later despite still efficient HDAC5 inhibition (Figure 2g). However, this reversible cell-cycle blocking was not observed in MCF-7 cells (data not shown).

To further investigate whether HDAC5 depletion altered cell-cycle progression, HDAC5-depleted cells were treated with the mitotic inhibitor nocodazole 24 h before harvesting
Addition of nocodazole resulted in accumulation of cells in M phase in mock- or GL3 siRNA-transfected cells. By contrast, HDAC5 depletion led to a decreased number of cells in M phase after nocodazole treatment, in favor of an accumulation of cells in both G1 and S phase. This demonstrated that HDAC5 depletion caused a defect in cell-cycle progression. However, after 48 or 72 h of transfection, HDAC5-depleted cells progressed through their cell cycle like

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control cells, suggesting that cells seem to adapt or recover from HDAC5 loss, thus ensuring normal cell-cycle progression (Figure 3).

**HDAC5 depletion inhibits replication fork progression.** The inability to remove histones in front of the replication fork or to load nucleosomes behind the fork can impede replication progression. To test whether HDAC5 depletion affected replication fork progression, we performed DNA fiber assay (Figure 4a). A comparison of DNA fibers from control- and HDAC5 siRNA-transfected cells revealed a striking difference in the overall length of their replication tracks (Figure 4b) and stalled replication fork were commonly observed when HDAC5 is depleted (Supplementary Figure S5). When distribution of fibers length was quantified and plotted, the entire distribution of fiber length in HDAC5-depleted cells shifted leftward to shorter fibers (Figure 4c). Quantification of the doubly labeled fibers indicated that the average rate of fork progression in HDAC5-depleted cells was 1.26-fold slower than in control cells, suggesting that replication forks progressed at a slower rate in the absence of HDAC5 (Figure 4d).

DNA replication is a multi-step process, which first requires loading of DNA replication licensing factors. The chromatin association of ORC (origin recognition complex) and MCM (mini-chromosome maintenance protein) subunits, and PCNA, which are DNA replication licensing factors—was not altered in the absence of HDAC5 (Figure 4e), suggesting that replication factors were assembled normally and the replication defect occurred downstream from replication factor recruitment.
Analysis of the activities of lamin B2, an early replication origin, and Ors8, a late replication origin, demonstrated that HDAC5 depletion affected the firing of origins. Indeed, firing from both origins was reduced by approximately 40% at 24 h after transfection, whereas it was significantly increased after 48 h of transfection (Figures 4f and g).

HDAC5 depletion induces DNA damages and activates DNA-damage checkpoint pathways. Changes in heterochromatin structure alter fork progression. DNA double-strand breaks (DSBs) arise frequently as a consequence of replication fork stalling. One of the first molecules to appear following DSB formation is the phosphorylated form of H2AX histone variant on Serine139 (γ-H2AX). Inhibition of HDAC5 expression led to a significant increase in γ-H2AX as early as 24 h after transfection as shown by western blotting and single-cell electrophoresis assay (Figures 5a and b), suggesting that HDAC5 depletion leads to DNA damages. A co-staining between γ-H2AX and BrdU revealed that these DNA damages occur at replication sites mainly in mid- to late S phase (Figure 5c and Supplementary Figure S6).

DNA damages during S phase activate the intra-S phase checkpoint and involve transducer kinases such as checkpoint kinase 1 (Chk1) and/or Chk2.15,16 The active form of Chk1 but not Chk2 was detected in HDAC5-depleted cells. The level of p53, a downstream target of Chk1, is slightly increased 24 h after HDAC5 depletion, but it declined thereafter, and reached the basal level after 32 h. In HDAC5-depleted cells, the p53 target gene p21WAF1/Cip1 also showed a similar rise and fall, although it faded off more slowly. Similarly, upregulation of p27kip1 and p16INK4A, two other cyclin-dependent kinase (CDK) inhibitors (CDKis), was transient and not sustained over time. As attempted, these CDKis inhibit the phosphorylation of retinoblastoma protein (pRb), thereby preventing E2F1 from transcribing genes that are required for cell-cycle progression such as cyclin A or E2F1 itself (Figure 5d). However, induction of these CDKis and accumulation of hypo-phosphorylated pRB were not maintained after 72 or 96 h (Figure 5e), showing that HDAC5 depletion caused a transient induction of CDKis that decline slightly at basal level later.

To see whether induction of both p53 and p21WAF1/Cip1 was similar in other cell types, we explored the consequences of...
**Figure 4** (a–d) HDAC5 depletion inhibits replication forks. (a) A schematic representation showing the principle of the DNA fiber assay. (b) Representative images of replication tracks in mock (No siRNA)- and HDAC5 siRNA-transfected cells pulse-labeled with 50 μM IdU for 20 min (green track) followed by 50 μM CldU for 30 min (red track), and then processed for DNA fiber spreads as described under Materials and Methods. Fork direction is indicated by a black arrow. All track photos are shown at identical magnifications (original magnification: ×630; bar: 5 μm). (c) The numbers of fibers for each specified length in mock (No siRNA)-, HDAC5 siRNA-, and GL3 siRNA-transfected cells were compared. The data were derived from one of two independent experiments in which at least 100 fibers were analyzed per experiment. Results are expressed as a frequency distribution of fiber length. Fiber length means were compared by one-way ANOVA with a 95% interval of confidence followed by Bonferroni’s post-test. (d) Mean fork rates (kb/min) ± S.D. in each condition were calculated from the data shown in panel c, and compared by one-way ANOVA with a 95% interval of confidence. (e) HDAC5 depletion does not alter the chromatin loading of DNA replication licensing factors. HeLa cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24 or 48 h. Fractions of chromatin-bound proteins were prepared as described under Materials and Methods, and the level of different DNA replication licensing factors was assessed by western blotting. The MEK2 protein was used as a fractionation control. S2, cytoplasmic fraction; S3, nuclear soluble proteins; P3, chromatin-enriched fraction. (f and g) HDAC5 depletion inhibits the firing of origins. Histogram plots of the lamin B2 (f) and Ors8 (g) origin activities in mock-transfected cells or cells transfected with a siRNA directed against either HDAC5 or GL3 for 24 or 48 h as measured by nascent-strand DNA abundance. Results are expressed as a percentage of the nascent-DNA strand abundance mean under the No siRNA condition. The values represent the mean ± S.D. of two independent experiments, each with three technical replicates. Statistical analysis was performed by two-way ANOVA with a 95% interval of confidence followed by Bonferroni’s post-test.

***P < 0.001
HDAC5 depletion in MCF-7 cells, which also harbors wild-type p53 and pRb genes. In those cells, HDAC5 depletion also induced activation of the p53–p21WAF1/Cip1 and pRb pathways, but the induction was more persistent compared with HeLa cells (Figure 5f).

**Figure 5** HDAC5 depletion leads to DNA damages and activates the DNA-damage checkpoint pathway. (a) HeLa cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24 h, and western blotting was performed using anti-γH2AX antibodies. (b) HeLa cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24 h, and the presence of DNA damages was assayed by the Oxiselect Comet Assay Kit according to the manufacturer’s instructions (Cellbiolabs, San Diego, CA, USA). Representative cells are shown for each condition. A higher number of nuclei in HDAC5-depleted cells showed the presence of the characteristic comet tail, indicating the presence of DNA damages. The arrows indicate cell nuclei with tail (original magnification: × 630; bar: 10 μm). The average comet tail moment length was scored for at least 100 nuclei per slide by using the CASP software, version 1.2.2 (www.casp.cf.pl). Results are expressed as mean ± S.D. and are indicated under the pictures. (c) HeLa cells were transfected as in panel a, pulse-labeled for 30 min with the nucleotide analog BrdU, fixed, and double immunostained for γH2AX (green) and BrdU (red). Representative pictures of HDAC5-depleted cells from two independent experiments are shown. The white arrows indicate colocalization foci into the nucleus. (d) HeLa cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24, 32 or 48 h. Total protein extracts were prepared and processed for western blotting using the indicated antibodies. HSC70 was used as a loading control. HeLa cells (e) or MCF-7 cells (f) were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24, 48, 72, or 96 h. Total protein extracts were prepared and processed for western blotting using the indicated antibodies. HSC70 was used as a loading control.
of senescence and/or induce apoptosis. Any blue staining indicative of SA-β-gal (senescence-associated β-galactosidase) activity, a typical marker of senescence, was observed in both HeLa and MCF-7 cells in the absence of HDAC5 (data not shown). However, HDAC5 depletion drives cells into apoptosis. By 48h after transfection, there was a nearly two-fold increase of cells undergoing apoptosis (Figures 6a and b). Apoptosis in both cell types was confirmed by caspase-7 activation (Figures 6c and d, and Supplementary Figures S7A and S7B) as well as microscopic analysis showing typical apoptotic morphology (Supplementary Figure S8).

As autophagy and apoptosis can share common components and inhibitory/activating signaling pathways, we next determined whether HDAC5 depletion could also induce autophagy by analyzing the level of LC3, an autophagosomal marker. LC3-II levels (compared with β-actin loading controls) increased in both HeLa and MCF-7 cells depleted for HDAC5 (Figures 6c and d, bottom panels), suggesting concomitant induction of apoptosis and autophagy. This amount of LC3-II further accumulates in the presence of a lysosomal inhibitor, indicating enhancement of the autophagic flux (Supplementary Figure S9A). To further confirm activation of autophagy, both HeLa and MCF-7 cells were stained with an LC3 antibody.

![Figure 6](image_url)

**Figure 6** HDAC5 depletion induces both apoptosis and autophagy. HeLa (a) or MCF-7 cells (b) were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24, 48, 72, and 96 h. Apoptotic cells were quantified by Annexin V staining as described under Materials and Methods. Results are presented as a relative number of apoptotic cells arbitrarily fixed as 1 under the No siRNA condition. The values represent the mean ± S.D. of three independent experiments. Statistical analysis was performed by two-way ANOVA with a 95% interval of confidence followed by Bonferroni’s post-test. ***P < 0.001. HeLa (c) or MCF-7 cells (d) were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24–48–72 or 96 h. Both floating and adherent cells were collected and lysed. Western blotting was performed using anti-HDAC5, anti-caspase-7, and anti-LC3 antibodies. β-Actin was used as a loading control. (e and f) Both HeLa and MCF-7 cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 48 h. Autophagic vacuoles (white arrows) were detected by confocal microscopy after LC3 staining. Representative cells are shown for each condition (original magnification: × 630; bar: 5 μm).
DNA replication in the context of chromatin, cell organization is crucial for eukaryotic cells. To orchestrate the inheritance and faithful maintenance of chromatin, scheduling of the combination of HDAC5 silencing with exposure to DNA-damaging drugs is required for each cancer cell type.

HDAC5 depletion globally reduces cancer cell proliferation, survival, and inhibits tumor growth in vivo. Because HDAC5 depletion affects cell-cycle progression, and induces both apoptosis and autophagy, we assessed the effect of HDAC5 depletion on the global cancer cell proliferation, survival, and tumor growth. In vitro, HDAC5 depletion significantly decreased proliferation (Figures 7a and b) as well as survival (Figures 7c and d) of both HeLa and MCF-7 cells. To examine the effect of HDAC5 depletion on tumor cell growth, we used an in vivo model in which cancer cells are engrafted onto embryonated chick chorioallantoic membrane (CAM). Tumors formed from HDAC5-depleted MCF-7 cells (Figures 7e–g) were smaller than control tumors, demonstrating the relevance of inhibiting HDAC5 in cancer cells in vivo.

HDAC5 depletion increases the efficacy of chemotherapeutic drugs in vitro. It has become increasingly clear that the chromatin compaction present in heterochromatin helps to protect DNA from damaging drugs.17 As such, knockdown of heterochromatic proteins or induced de-condensation of chromatin sensitizes cells to DNA damages.18 According to our data, HDAC5 depletion could expose heterochromatic regions to DNA-damaging drugs. Therefore, we examined whether HDAC5 depletion could sensitize cancer cells to chemotherapeutic drugs. MCF-7 or HeLa cells were transfected with a siRNA against HDAC5 for 24 h, and then incubated either with doxorubicin or cisplatin for an additional 24 h. Loss of HDAC5 caused 5–6 times more apoptosis compared with cisplatin or doxorubicin alone in HeLa cells (Figures 8a–c). Interestingly, the combination of HDAC5 siRNA/doxorubicin or cisplatin produces more cell death than chemotherapeutic drugs associated with trichostatin A (TSA), a broad-spectrum HDACi (Supplementary Figures S10A and S10B), demonstrating that only inhibition of HDAC5 produces better cytotoxicity than HDACi to potentiate chemotherapeutic drugs.

Using the same schedule, HDAC5 depletion in MCF-7 cells did not potentiate the activity of chemotherapeutic drugs. To test whether this schedule alters the effectiveness of combination treatment, HDAC5-depleted MCF-7 cells were exposed to DNA-damaging drugs 48 h after siRNA transfection for an additional 24 h. A better cytotoxic/apoptotic effect was observed (Figures 8d and e, and Supplementary Figure S10C) suggesting that appropriate sequencing and scheduling of the combination of HDAC5 silencing with DNA-damaging drugs is required for each cancer cell type.

Discussion
The inheritance and faithful maintenance of chromatin organization is crucial for eukaryotic cells. To orchestrate DNA replication in the context of chromatin, cells have evolved efficient nucleosome dynamics involving assembly pathways and chromatin maturation mechanisms. During replication, modified parental histones are displaced ahead of the replication fork and are randomly distributed between the two daughter strands. Concomitantly, deposition of de novo-synthesized histones H3 and H4 provides the full complement of histones that are needed to ensure proper assembly of the duplicated material. During its initial synthesis, histone H4 is acetylated at lysine residues 5 and 12. These residues must be deacetylated to form heterochromatin in late S phase, thus ensuring secure maintenance of the under-acetylated state of heterochromatin. This latter step is promoted through the action of HDAC-containing complexes such as Mi-2/NuRD (nucleosome-remodeling deacetylase complex) and/or the Sin3/HDAC chromatin-modifying complex,19 which have been shown to contain both HDAC1 and HDAC2, and associate with pericentric heterochromatin during S phase.20,21 Whereas some reports pointed to the role of HDAC2 in the rearrangement of the nucleosomes during the formation of heterochromatin in late S phase,22 as well as the implication for HDAC3 in replication fork progression,23,24 we demonstrated that HDAC5 is recruited to heterochromatin regions, probably with members of NuRD/Sin3 chromatin remodeling complexes as well as with other epigenetic regulators such as DNA methyltransferase 1 (DNMT1) or HP-1,22,25–28 to participate in the establishment of the pericentric heterochromatin structure in late S phase. However, the mechanisms of action of HDAC5 in the assembly/maturaton of pericentric heterochromatin remain to be identified.

The chromatin structural defect caused by HDAC5 depletion results in a slow-growth phenotype, with delayed cell-cycle progression and activation of multiple checkpoints pathways. We indeed observed: (i) activation of the Chk1-dependent intra-S phase checkpoint, which could survey alterations in chromatin structure, and serves as an efficient mechanism to slow down fork progression in the absence of appropriate chromatin assembly and block initiation of new replication forks in a global manner, consistent with reports showing that origin firing is inhibited during S phase when DNA damage or replication fork stalling activates the intra-S-phase checkpoint kinases,29–31 and (ii) activation of a G1/S checkpoint pathway, which prevents late-G1 cells from entering the S period by directly or indirectly inhibiting initiation at the earliest-firing origins, with the consequence that the entire S period is delayed along the cell-cycle axis.29,30

In addition to cell-cycle blocking, we also observed activation of autophagy in HDAC5-depleted cells. Numerous studies suggest that autophagy may function in the regulation of cell survival and have a major role in the maintenance of genomic integrity.32 The precise role of autophagy in response to HDAC5 depletion is not fully understood yet but is thought to be a temporary survival mechanism, which delays apoptosis as inhibition of HDAC5 silencing-induced autophagy unmasks and accelerates apoptosis (data not shown).

Activation of checkpoint pathways and consequently cell-cycle blocking in HDAC5-depleted cells should allow time for the chromatin defects to be resolved. Once repair is accomplished, these checkpoint/repair pathways are usually
silenced so that cell-cycle progression is allowed to resume. In HDAC5-depleted HeLa cells, we noticed resumption of normal cell-cycle progression after transient cell-cycle blocking as demonstrated by biphasic modulation of cell-cycle inhibitors such as p21\(^{WAF1/Cip1}\), p27\(^{Kip1}\), or P-pRb, which occurs in conjunction with release from cell-cycle blocking.
This transient cell-cycle arrest suggests that either checkpoint recovery (fulfillment of the requirement) or checkpoint adaptation occurs in HDAC5-depleted HeLa cells. In the light of an yeast two-hybrid screen showing interaction between HDAC5 and Mus81 (methyl methanesulfonate and ultraviolet-sensitive gene clone 81), we are actually investigating the role of this endonuclease Mus81 on cell-cycle resumption. Preliminary experiments demonstrated the possible implication of Mus81 in the checkpoint recovery/adaptation of cells from HDAC5 depletion stress, allowing them to survive and proliferate with apparently intact chromosomes or at the cost of tolerating mutation. As defective DNA-damage repairs with Mus81 mutations are often observed in breast cancer patients, we hypothesized that MCF-7 cells harbor mutations in gene encoding Mus81 and/or other DNA repair/checkpoint proteins that impair checkpoint recovery/adaptation,

Figure 8  HDAC5 depletion sensitizes both HeLa and MCF-7 cells to chemotherapeutic agents. (a and b) HeLa cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24 h and then treated either with doxorubicin (2 μM) (a) or cisplatin (7 μM) (b) for an additional 24 h. Apoptotic cells were quantified by Annexin V staining as described under Materials and Methods. ***P < 0.001. (c) HeLa cells were transfected as in panels a and b. Both floating and adherent cells were collected and lysed. Western blotting was performed using anti-caspase-7 and anti-LC-3 antibodies. HSC70 was used as a loading control. (d) MCF-7 cells were mock-transfected (No siRNA) or transfected with a siRNA directed against either HDAC5 or GL3 for 24 or 48 h, and then treated with doxorubicin (2 μM) for an additional 24 h. WST-1 cell survival assay was performed as described under Materials and Methods. (e) MCF-7 cells were transfected as in panel d. Apoptotic cells were quantified by Annexin V staining. For each condition, representative FACS dot plots are presented with number of cells in Annexin V⁻/PI⁻ quadrant
leading to excessive unrepaired chromatin defects and consequently apoptosis.

After an initial attempt to recover or adapt, prolonged HDAC5 inhibition in HeLa cells overwhelms the cells and results in apoptosis, suggesting that either chromatin defects are too severe or recovery/adaptation mechanisms are followed by excessive genome instability, leading to cell death in subsequent cell cycles for a subpopulation of cells. In addition to activation of the apoptotic program, we noted that autophagy is still induced at late time point in the time course of HDAC5 depletion. Despite its initial role as a survival pathway, progressive autophagy can result in cell death if allowed to proceed to completion under persistent stress and therefore, both processes would cooperate to lead to cell death.

The role of HDAC5 in heterochromatin assembly and maturation also has an impact in DNA alteration-based cancer strategies. Consistent with others’ studies, we reported here that HDAC5 depletion potentiates the effect of chemotherapeutic agents that target DNA by inducing heterochromatin de-condensation, thereby facilitating access of drugs to DNA. Despite a more accessible chromatin in both cell types, we found that a different sequencing and scheduling of the combination of HDAC5 silencing with DNA-damaging drugs is required for each cancer cell types. Why did HDAC5-depleted MCF-7 cells show a delayed death response to DNA-damaging drugs compared with HeLa cells? In MCF-7 cells, several factors such as caspase-3 deficiency, levels and activity of the Bax (Bcl-2-associated X)/Bcl-2 (B-cell lymphoma 2) proteins, or activation of phosphoinositide-3-kinase (PI3K)/Akt (protein kinase B) kinases can contribute to refractory to apoptosis induced by DNA-damaging drugs. Autophagy also represents a mechanism of resistance to modalities, which affect DNA. Despite a more accessible chromatin in both cell types, we found that a different sequencing and scheduling of the combination of HDAC5 silencing with DNA-damaging drugs is required for each cancer cell types.

Materials and Methods

Cell culture, synchronization, and treatment. HeLa cells were maintained in Dubbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS). MCF-7 cells were maintained in x-Modified Eagle’s Medium (xMEM) supplemented with 10% heat-inactivated FBS.

Synchronization was achieved by treating cells with 2 mM hydroxyurea (Sigma) and 200 μg/ml nocodazole (Sigma, St. Louis, MO, USA). TSA, cisplatin and doxorubicin were purchased from Sigma.

Antibodies. Anti-FLAG and anti-BrdU (clone BU-33) antibodies were purchased from Sigma. Anti-BrdU (clone BU17/75), anti-ORC1, anti-ORC3, and anti-ORC4 antibodies were from AbDserotec (Kidlington, UK). Anti-HDAC5, anti-p16NK4A, anti-MCM2, anti-MEK2 (MAPK/ERK kinase 2), anti-ORC2, anti-Chk1, anti-phosphoSer317 Chk1, anti-Chk2, anti-phosphoThr68 Chk2, and anti-caspase-3/7 antibodies were purchased from Cell Signaling (Carfabad, CA, USA). Anti-HDAC5, anti-histone H2AX, anti-phosphoSer139 histone H2AX and anti-phosphoSer10 histone H3 antibodies were from Millipore (Bedford, MA, USA). Anti-BrdU (clone B44), anti-MCM4, anti-MCM5, anti-MCM6, anti-p27, anti-pRb, anti-cyclin E, and anti-cyclin A antibodies were purchased from BD Biosciences (Eremboogem, Belgium). Anti-p53 antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-p21, anti-E2F1, anti-MCM3, anti-Cdc6, anti-PCNA (clone PC10), anti-HP1, anti-HDAC5, and anti-HSC70 (heat-shock cognate 70-kDa protein) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-LC3 antibodies were purchased from Abgent (San Diego, CA, USA).

siRNA transfection. siRNAs were synthesized either by Eurogenc (Liège, Belgium) or Dharmaco (Lafayette, CO, USA). Calcium phosphate-mediated transfections were performed as described previously.

Immunocytochemistry. After fixation and permeabilization, cells were incubated with primary antibodies and with corresponding Alexa dye-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) and mounted onto microscope slides. For nuclear counterstaining, cells were incubated with TOPRO-3 (Molecular Probes). For γ-H2AX and BrdU co-staining, cells were fixed and permeabilized. To denature DNA, fixed cells were resuspended in 4 N HCl and incubated for 30 min at 37°C. After washing with borate buffer and phosphate-buffered saline (PBS) to remove any acid traces, cells were simultaneously incubated with a mouse anti-BrdU antibody and a rabbit anti-γ-H2AX antibody. Primary antibodies were detected with a secondary Alexa 546-conjugated goat, anti-mouse antibody and a secondary Alexa 488-conjugated goat, anti-rabbit antibody. Images were obtained with either a Leica TCS SP5 laser-scanning confocal microscope (Leica, Wetzlar, Germany) or with a Fluoview Olympus laser-scanning confocal microscope (Olympus, Tokyo, Japan). Images were transferred to Adobe Photoshop CS4 (Adobe Systems) for assembly.

Transmission electron microscopy. Immunolabeling was performed on formaldehyde-fixed and Lowryl-K/M-embedded cells as described previously. For ultrastructure, samples were washed in Sörensen’s buffer and fixed for 1 h at 4°C with 2.5% glutaraldehyde in Sörensen’s 0.1 M phosphate buffer (pH 7.4), and post-fixed for 30 min with 1% osmium tetroxide. After dehydration in graded ethanol, samples were embedded in Epon. Ultrathin sections obtained with a Reichert Ultracut S ultramicrotome were contrasted with uranyl acetate and lead citrate. Observations were made with a Jeol 100 CX II transmission electron microscope at 60 kV.

MNs sensitivity assay. MCF-7 cells were Dounce-homogenized in RSB buffer (10 mmol/l Tris-HCl (pH 7.4), 10 nM NaCl, 3 mM MgCl2, 0.5% NP-40) containing 10 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 1 mM dithiothreitol (DTT), and incubated on ice for 15 min. Samples were centrifuged at 4°C for 5 min at 14,000 g. The medium was removed, samples were washed twice with RSB buffer, and digested with 0.25 to 2.5 U of micrococcal nuclease in digestion buffer (15 mM Tris-HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 1 mM CaCl2, 3 mM MgCl2, 20% glycerol, 15 mM β-mercaptoethanol) for 5 min. Digestion was stopped by adding 1 volume of stop solution (50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM EDTA, 0.3% sodium dodecyl sulfate (SDS)). DNA was extracted using 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by 1 volume chloroform/isoamyl alcohol (24:1) and precipitated with 100% ethanol. DNA was washed once with 70% ethanol, resuspended in H2O, and 1 μg of DNA was separated using 1% agarose gel.

Chromatin isolation. Chromatin fractionation was performed as described previously. Three different fractions were collected: cytoplasmic fraction (S2), nuclear soluble proteins (S3), and chromatin-enriched fraction (P3).

Western blot analysis. Adherent (and floating depending on the experiment) cells were lysed into an SDS buffer (SDS 1%, Tris-HCl 50 mM, protease inhibitor mixture) unless otherwise stated. Equal amounts of proteins were resolved by SDS-PAGE. Membranes were probed with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies, and developed by chemiluminescence detection.

WST-1 cell viability. Cell survival was determined by WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) cell viability assay according to the manufacturer’s instructions (Roche, Basel, Switzerland).
Cell-cycle analysis. The relative percentage of cells in each stage of the cell cycle was analyzed according to the procedure of labeling nuclei with propidium iodide (PI) followed by flow cytometric analysis using FACS Calibur II and the ModFit LT program.

**In vitro DNA content measurement.** Fluorometric DNA titration was performed as described previously.26

**In vivo DNA replication assay.** Cells were labeled with 33 µM BrdU for 30 min, resuspended in PBS in 10% PBS, and then fixed with 70% cold ethanol. DNA was denatured with 4 N HCl + 0.5% Triton X-100 for 30 min at 37°C. After washing cells with PBS, the pellet was resuspended in PBS + 10% PBS. An anti-BrdU antibody (clone BU-33; Sigma) was added and cells were incubated for 1 h at room temperature. After washing, an Alexa 488-conjugated goat, anti-mouse secondary antibody (Molecular Probes) was added for 1 h at room temperature in dark. After washing, cells were collected, resuspended in PI solution (EDTA 3 mM (pH 8.0). Tween 20 0.05%, PI 50 µg/ml, RNase A 50 µg/ml in PBS), and analyzed using a FACS Calibur II and the CellQuest software (BD Biosciences).

**DFA fiber assay.** Cells were doubly labeled by incubating with 50 µM iododeoxyuridine (IdU) for 20 min followed by incubation with 50 µM chlorideoxygenidourine (CldU) for 30 min. A 2-µl volume of resuspended, in ice-cold PBS at 105 cells/ml, was spotted onto a silane-coated microscope slide (Sigma) and then overlaid with 10 µl of spreading buffer (SDS 0.5%, Tris-HCl 200 mM (pH 7.4), EDTA 50 mM). After 6 min, the slides were tilted by 15 degrees to allow lysates to slowly move down the slide. The DNA spreads were air-dried, fixed in a 3:1 mixture of methanol/acetic acid (15 min at –20°C) and stored in pre-chilled 70% ethanol at 4°C overnight. The slides were then treated with 4 M HCl for 10 min at room temperature followed by 30 min at 37°C in a water bath, washed three times in PBS, and incubated in blocking buffer (2% BSA in PBS) for 1 h at room temperature followed by 1 h at 37°C with a rat anti-BrdU antibody (to detect CldU) (clone BU1/75; AbDSerotec) plus a mouse anti-BrdU (to detect IdU) (clone B44; BD Biosciences). After rinsing three times with stringency buffer (Tris-HCl 10 mM (pH 7.4), EDTA 50 mM), the slides were incubated for 1 h with Alexa Fluor 488-conjugated rabbit, anti-mouse antibodies and Alexa Fluor 546-conjugated goat, anti-rat antibodies (Molecular Probes). Slides were rinsed three times with PBS, once with H2O, and mounted in Mowiol medium. Microscopy was performed using a Fluoview Olympus laser-scanning confocal microscope using the sequential scanning mode. A single-blind evaluation was performed to measure the lengths of continuously double-stained tracks using the ImageJ software (NIH, Bethesda, MD, USA) and the collected images were processed using the Adobe Photoshop CS4 software (Adobe Systems). Pictures were taken on the entire slide and on multiple slides, reducing the chance of over- or under-representing certain origins or genomes of individual cells. Micrometer values were converted into kilobase using the conversion factor 1 µm = 0.259 kb. Measurements were recorded from fibers in well-spread (untangled) areas of the slides to prevent the possibility of recording labeled patches from bundles of fibers.

**Isolation of nascent-strand DNA.** Isolation of nascent-strand DNA was performed as described previously.26 The primers and PCR conditions are described by Rampaakakis et al.26

**Annexin V staining.** Apoptotic cells were determined by Annexin V–FITC (fluorescein isothiocyanate) and non-vital dye PI staining using an FITC–Annexin V apoptosis detection kit I (BD Biosciences) according to the manufacturer’s instructions. Flow cytometry was performed using a FACS Canto and samples were analyzed using the CellQuest software (BD Biosciences). Both Annexin V+PI– cells representing early-apoptotic cells and Annexin V+PI+ mostly representing late-apoptotic/necrotic cells were considered as apoptotic cells.

**CAM tumor model.** A window was opened in the eggshell of a 3-day-old embryo using scissors after puncturing the air chamber. The window was sealed with tape and the eggs were incubated at 37°C and 80% humidity until cell grafting. Eight days later, a Matrigel/cell mixture (1:1) was grafted within a plastic ring on the CAM surface. The window was sealed and the eggs were incubated under the same conditions for 7 days. On day 18, tumors were dissected and diameters were measured with a Vernier caliper. Tumor volume was calculated using an ellipsoid formula:

\[
\text{Volume (mm}^3\text{)} = \frac{4}{3} \times \pi \times Z_1 \times Z_2 \times Z_3
\]

where \(Z_1, Z_2, Z_3\) are the main radii of the tumor.

**Statistical analyses.** Results were reported as means with their S.D. or S.E.M. as reported in the figure legends. Statistical analysis was performed by one-way ANOVA or two-way ANOVA regarding the number of grouping factors. Group means were compared by Bonferroni’s post-test. Homoscedasticity was assessed by Levene’s test. Normality was assessed by the D’Agostino and Pearson test. All tests were performed with a 95% interval of confidence.

**Conflict of Interest**

The authors declare no conflict of interest.

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Revealing the anti-tumoral effect of Algerian Glaucium flavum roots against human cancer cells.


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Revealing the anti-tumoral effect of Algerian *Glaucium flavum* roots against human cancer cells

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

\textit{Glaucium flavum} (\textit{G. flavum}) is a plant from the Papaveraceae family native to Algeria where it is used in local traditional medicine to treat warts. \textit{G. flavum} root crude alkaloid extract inhibited breast cancer cell proliferation and induced G2/M phase cycle arrest and apoptosis without affecting normal cells, which is a highly awaited feature of potential anti-cancer agents. \textit{G. flavum} significantly reduced growth and vascularization of human glioma tumors on chicken chorioallantoic membrane (CAM) in vivo. The chromatographic profile of the dichloromethane extract of \textit{G. flavum} root showed the presence of different constituents including the isoquinoline alkaloid protopine, as the major compound. We report for the first time that \textit{G. flavum} extract may represent a new promising agent for cancer chemotherapy.

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\textbf{Introduction}

Breast cancer is one of the most prevalent malignancies in women in many countries worldwide (Jemal et al. 2011; Youlden et al. 2012). After the rapid expansion of the use of monoclonal antibodies and various synthetic inhibitors directed against matrix metalloproteases or protein kinases, natural products are regaining attention in the oncology field. Due to their wide range of biological activities and low toxicity in animal models, natural products have been used as alternative treatments for cancers including breast cancer. An analysis of new and approved drugs for cancer by the United States Food and Drug Administration (FDA) over the period 1981–2010 showed that more than half of cancer drugs were of natural origin (Newman and Cragg 2012).

Cell cycle deregulation resulting in uncontrolled cell proliferation is one of the most frequent alterations that occur during tumor development. For this reason, blockade of the cell cycle is regarded as an effective strategy for eliminating cancer (Lapenna and Giordano 2009; Williams and Stoebber 2012). Key regulator proteins are cyclin-dependent kinases which activity is specifically controlled by cyclins and cyclin-dependent kinase inhibitor (CDKI) at specific points of the cell cycle (Besson et al. 2008). The G2/M checkpoint is the most conspicuous target for many anticancer drugs. P21, a member of the CDKI family and cyclin B1 are the central players of G2/M phase transition (Vermeulen et al. 2003). There is a tight relationship between the control of cell cycle checkpoints and the progression to apoptosis, a mechanism responsible for maintaining tissue homeostasis by mediating the equilibrium between cell proliferation and death. Defective apoptosis represents a major causative factor in the development and progression of cancer (Cotter 2009; Ricci and Zong 2006). In cancer therapy, induction of apoptosis cells is one of the strategies for anticancer drug development (Alam 2003; Fischer and Schulze-Osthoff 2005; Ocker and Höffner 2012).

Several drugs currently used in chemotherapy were isolated from plant species. The best known are the Vinca alkaloids, vinblastine and vincristine, isolated from \textit{Catharanthus roseus}, etoposide and teniposide, which are semi-synthetic derivatives of the natural product epipodophyllotoxin, Paclitaxel isolated from the bark of \textit{Taxus brevifolia}, the semi-synthetic derivatives of campothecin, irinotecan and topotecan, isolated from \textit{Camptotheca acuminata}, among several others (Cragg et al. 1993).

\textit{G. flavum} belongs to the family of Papaveraceae. The aerial part of this plant is very rich in isoquinoline alkaloids, especially in aporphine bases namely, didehydroglaucine, 6',7'-dehydroyglaucine,(+)–glaucine, (+)–isocorydine, (+)–corydine, (+)–cataline, 1,2,9,10-tetramethoxyxooaporphine, \(\alpha\)–allocryptopine, corurnine, and isoboldine (Israelov et al. 1979; Daskalova et al. 1979;...
1988). The latter are known for exhibiting promising pharmacological activities including anti-inflammatory, analgesic and antipyretic (Pinto et al. 1998), hypoglycemic (Cabo et al. 2006) and antioxidant activity (Tawaha et al. 2007).

Interestingly, a recent study demonstrated that Sardinian G. flavum contained a homogeneous alkaloid pattern of aporphine type, significantly different from those reported for populations from other parts of Europe (Petitto et al. 2010). In this study, we used G. flavum collected in Algeria where the root is widely used in local traditional medicine to treat warts and inflammatory diseases. To our knowledge, the potential anticancer activities of G. flavum have never been investigated. We decided to evaluate the effects of its alkaloid extract on human normal and malignant cells.

We explored the potential inhibitory growth effect of the dichloromethane extract of G. flavum root on 3 human breast cancer cell lines: MDA-MB-435, MDA-MB-231 and Hs578T and non malignant human cells. Interestingly, G. flavum induced cell cycle arrest and apoptotic cell death in all breast cancer tested cells but not in MCF10A normal mammary epithelial cells. Based on our results in vitro, we decided to explore further the anti-tumoral effect of G. flavum extract using the in vivo tumor chorioallantoic membrane (CAM) model. We demonstrated that G. flavum extract treatment induced a significant decrease in tumor growth and affected tumor associated angiogenesis in vivo. The chemical characterization of the dichloromethane extract was evaluated using HPLC analysis, which showed the presence of protopine as the major alkaloid.

Materials and methods

Plant material extraction

The root of flowering plant G. flavum was collected in littoral area and far from any contact with pollution in Tichy, province of Bejaia (Algeria) according to botanists (University of Bejaia) previous identification. The alkaloids were extracted as described by Suau et al. (Suau et al. 2004). Briefly, the extraction was undertaken with (10 g) of powdered plant material and (100 ml) of methanol in a Soxhlet apparatus. The methanol was evaporated using a rotavapor and the residue was taken up in 2% hydrochloric acid (50 ml), neutral components being removed by filtration. The filtrate was adjusted to pH 8 with aqueous ammonia and extracted with dichloromethane (3×25 ml). The resulting extracts were dried with MgSO4 and the solvent evaporated to obtain the crude alkaloid extract. The solid extract was reconstituted in DMSO solvent (50 mg/ml stock solution) and then filtered using 0.22 μm filters before storage at −20°C. During all experiments, DMSO dilutions of C. flavum extract were adjusted in the culture media to achieve the indicated final concentrations and control cells were treated at the maximum concentration used in the experiment, 0.1%.

High performance liquid chromatography (HPLC) profiling

The HPLC of dichloromethane root extract of G. flavum (stock solution 20 mg/50 ml) was carried out for identification. The extract was dissolved in methanol and filtered through Acrodisc PSF GXF/GHP 0.45 nm filter. An injection of 10 μl of this filtered extract was chromatographed with an Agilent 1100 HPLC with DAD (diode–array detector) detection. The working wavelength was 290 nm. The column was a Polaris amide C-18 column (5 μm, 250 mm × 4.6 mm) operated at 25°C. The mobile phase was composed of solution A (trifluoroacetic acid 0.05% in water) and solution B (acetonitrile) with the following gradient: equilibration time 15 min at 100% A and linear gradient elution: 0 min 100% A; 1 min 97% A; 45 min 60% A; 55 min 40% A and 65 min 100% A. The flow rate was 1 ml/min. The structure of protopine was elucidated using NMR spectroscopy (1H, 13C, COSY, HMBC, HSQC), mass spectrometry (MS), and UV spectroscopy.

Cell culture

MCF10A cells (CRL-10317, ATCC) were cultivated in DMEM/HAM’S F-12 medium supplemented with 0.01 mg/ml of human insulin, 2.5 μM l-glutamine, 20 ng/ml of epidermal growth factor, 0.5 mg/ml of hydrocortisone, 5% horse serum, and 100 ng/ml of cholera toxin. HUVEC (Human Umbral Vein Endothelial) and skin fibroblast were isolated and maintained in culture as described previously (Jaffe et al. 1973; Rittié and Fisher 2005). MDA-MB-231 (HTB-26, ATCC), MDA-MB-435 (HTB-129, ATCC) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% of fetal bovine serum and 1% l-glutamine. Hs578T cells (HTB-126, ATCC) were cultured in DMEM supplemented with 10 μg/ml of bovine insulin, 1 mM sodium pyruvate and 10% of fetal bovine serum. Human glioma cells U87-MG (89081402, ATCC) were maintained in Minimum Essential Medium with 10% FBS, 2 mM l-glutamine, 1% non essential amino acid, and 1 mM sodium pyruvate. All the cells were cultured at 37°C in a humidified atmosphere and 5% CO2.

Viability assay

Cell viability was determined using the cell proliferation reagent WST-1 assay according to the manufacturer’s instructions (Roche, Basel, Switzerland). All analyzed cells were seeded to obtain 50% of confluence after 24 h of incubation in 96-well plates then treated with serial dilutions of the plant extract (0–40 μg/ml). Cells were incubated with the WST-1 reagent for 4 h. After this incubation period, the formazan dye formed is quantified with a scanning multi-well spectrophotometer at 450 nm. The measured absorbance directly correlates to the number of viable cells. Percentages of cell survival were calculated as follows: % cell survival = (absorbance of treated cells/absorbance of cells with vehicle solvent) × 100. The half inhibitory concentration (IC50) was calculated from the dose-response curve obtained by plotting the percentage of cell survival versus the concentration of plant extract used.

Cell cycle analysis

Cells were seeded and incubated overnight to attach, and exposed to DMSO (control) or desired concentrations of G. flavum for specified time periods. Both floating and adherent cells were collected, washed with phosphate buffered saline (PBS), and fixed in 70% ethanol. The cells were then treated with 50 μg/ml RNase A and 50 μg/ml propidium iodide for 30 min and analyzed using a FACS Calibur II and the Cell ProQuest program.

Antibodies

Anti-p21 and anti-cyclin B1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-beta actin from Sigma (Saint Louis, Missouri, USA).

Immunoblotting analysis

Desired cell line was seeded in 6 well plates, allowed to attach overnight and treated according to their respective IC50 with G. flavum extract. Both floating and attached cells were collected and lysed into an SDS buffer (SDS 1%, Tris–HCl 40 mM (pH 7.5), EDTA 1 mM, protease inhibitor mixture). Protein concentration was determined using a BCA kit according to manufacturer’s
instructions (Pierce, Rockford, IL). Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose or PVDF membrane (Invitrogen). Membranes were probed with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies, and developed by chemiluminescence detection. Blots were stripped and re-probed with anti-actin to normalize. Scanned bands were quantified using ImageJ software Version 1.43 (National Institutes of Health, http://rsb.info.nih.gov/ij/).

DAPI staining

DAPI staining method was used to observe apoptotic morphological changes (chromatin condensation and nuclear fragmentation) in treated cells. Cancer cells were treated with *G. flavum* extract at their respective IC50 values and MCF10A breast cells were treated at the highest IC50 observed for cancer cells (15 μg/ml) during 24 h. Briefly, the cells were seeded in 6 well plates and treated with *G. flavum* or with nocodazole (3 μM) used as a positive control of apoptosis inducer. Cells were fixed with paraformaldehyde 3% and incubated in Vectashield solution (Vector Laboratories) for 30 min in the dark. Cells were then examined and photographed using a fluorescence microscope (EVOS, AMG).

Quantitation of apoptosis by flow cytometry

Apoptotic cells were determined by Annexin V-FITC (fluorescein isothiocyanate) and non-vital dye PI staining using an FITC-Annexin V apoptosis detection kit I (BD Biosciences) according to the manufacturer’s instructions. Flow cytometry was performed using a FACS Caliber II and samples were analyzed using the CellQuest software (BD Biosciences).

Tumor CAM assay

The implantation of human glioblastoma U87-MG cells on the chorio-allantoic membrane (CAM) of embryonic chicken was performed as we described previously (Lamour et al. 2010). On day 13, size-matched tumors were divided into control and treatment groups. *G. flavum* extract was deposited locally at 100 μg/ml per egg per day. Digital pictures were taken under a stereomicroscope (Leica). On day 17, tumor size was calculated based on tumor volume formula: \[ V = (d_1/2)^3 + (d_2/2) * (d_3/2) * 3.14 * 4/3, \] with
Fig. 2. *G. flavum* extract inhibited the viability of malignant human breast cancer cells (MDA-MB-435, MDA-MB-231, Hs578T) in a dose-dependent manner without affecting normal human cells (MCF10A human normal mammary cells, human skin fibroblasts and HUVEC); (A) cells were treated with DMSO vehicle or the indicated concentrations of *G. flavum* extract for 24 h. Cell viability was determined using Wst1 assay and expressed as means ± SD of at least two separate experiments and (B) IC50 values of *G. flavum* extract were determined based on the dose-response curves shown in (A) (means ± SD of at least two separate experiments).

$d_1$, $d_2$, $d_3$ corresponding three measures taken on the experimental tumors. Quantification of drug effects on tumor cell growth were determined in ten representative tumors per group. For histological studies, U87-MG experimental tumors were embedded in paraffin and cut into 5 μm sections. Tissue sections were stained with hematoxylin and eosin (H&E). Vessels were stained using fluorescein-coupled *Sambucus nigra* lectin SNA-1 (FL-1301; Vector Laboratories). Statistical comparison between the two groups was performed by using the Student’s t test. A value of $p < 0.05$ was considered significant.

Results

Phytochemical profile of *G. flavum* extract

The HPLC chromatogram of *G. flavum* root extract (Fig. 1A) revealed several peaks with a significant peak eluting at 24.85 min (peak 1) which is the major compound of dichloromethane extract and was identified as protopine. Fig. 1B shows the structure of protopine.

*G. flavum* extract treatment specifically decreased the viability of human breast cancer cells

We first investigated the effects of *G. flavum* extract on MDA-MB-231, MDA-MB-435 and Hs578T human breast cancer cell lines. WST1 viability assay was used for the determination of *G. flavum* extract IC50 values in these cells thus establishing the starting point for the next experiments. Cells were treated during 24 h at the following concentrations: 0, 2.5, 5, 10, 20 and 40 μg/ml (Fig. 2A). The treatment significantly affected the viability of all cancer cell lines tested and displayed low IC50 values (<15 μg/ml) after 24 h of treatment (Fig. 2B). Following the standard National Cancer Institute (NCI) criteria, an IC50 less than 30 μg/ml of crude extract is considered as an active compound against cancer cells (*Suffness and Pezzuto 1990*). Next, we tested the effect of *G. flavum* extract on non malignant human cells such as spontaneously immortalized breast epithelial cells (MCF10A), human umbilical vein endothelial cells (HUVEC) and skin fibroblasts (Fig. 2A). For these cells, the calculated IC50 values were higher than 30 μg/ml indicating that *G. flavum* extract mainly inhibited breast cancer cell viability without affecting normal cells (Fig. 2B).

*G. flavum* extract treatment caused G2/M phase cell cycle arrest in human breast cancer cells

Next, we tested whether inhibitory effect of *G. flavum* on breast cancer cell viability was due to perturbations in cell cycle progression. Fig. 3 depicts flow cytometry histograms for cell cycle distribution in *G. flavum*-treated MDA-MB-231, Hs578T and MCF10A cells. After 24 h, *G. flavum* treatment (IC50) resulted in statistically significant enrichment of G2/M phase cell population in MDA-MB-231 cells as compared with DMSO-treated control cells (44.4% and 19.5%, respectively). In these cells, *G. flavum* mediated G2/M phase cell cycle arrest accompanied by a significant decrease in G0/G1 (from 62.8% to 26.5%) and S phase cells (from 16.5% to 12.7%). With time, a major increase from 0.6% to 16.2% in subG1 population was observed and corresponded to cells that have lost some of their DNA in late stages of the apoptotic process following endonucleases activity (Fig. 3). Notably, *G. flavum*-treated Hs578T cells presented with similar cell cycle pattern (Fig. 3). Consistent with the cell viability experiment, *G. flavum* extract used at the highest cancer cells IC50 value (15 μg/ml) did not affect cell cycle distribution of MCF10A cells and stability was generally observed in all cell cycle subpopulations after 12 and 24 h of treatment (Fig. 3).

*G. flavum* extract treatment altered the expression level of proteins involved in the regulation of G2/M transition in MDA-MB-231 cells

To gain insight into the mechanism of G2/M phase cell cycle arrest, we determined the effect of *G. flavum* treatment on the expression of proteins known to be involved in the regulation of G2/M transition. The level of p21 protein was increased after 12 h.

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Fig. 3. *G. flavum* extract treatment caused G2/M phase cell cycle arrest in MDA-MB-231 and Hs578T cells. Representative histograms depicting cell cycle distribution in Hs578T, MDA-MB-231 and MCF10A cultures following 12 h and 24 h treatment with DMSO vehicle or the indicated concentrations of *G. flavum* extract corresponding to IC₅₀ for Hs578T, MDA-MB-231 and to the highest cancer cells IC₅₀ (15 μg/ml) for MCF10A. The experiment was performed three times.

of treatment with *G. flavum* extract and was still high after 48 h. Interestingly, *G. flavum* treatment caused an early increase in the level of cyclin B1 that was sustained after 24 h and showed a slight decrease at 48 h (Fig. 4).

*G. flavum* extract treatment induces apoptosis in breast cancer cells in vitro

Staining of cells with DAPI showed morphological features characteristic of apoptotic cells such as DNA fragmentation and condensation of chromatin in breast cancer cells treated with *G. flavum* extract (IC₅₀, 24 h) that were comparable to nocodazole treated cells used as control (Fig. 5A). Untreated breast cancer cells and MCF10A cells treated with *G. flavum* extract exhibited a normal nuclear morphology characterized by large nuclei with distinguishable nucleoli and diffused chromatin structure (Fig. 5A). The quantification of apoptosis was next evaluated by annexin-V/propidium iodide (PI) staining. Dual staining with annexin-V and PI allowed clear discrimination between unaffected cells (annexin-V negative and PI negative), early apoptotic cells (annexin-V positive and PI negative) and late apoptotic cells (annexin-V positive and PI positive). Immunoblotting for each protein was performed at least three times using independently prepared lysates.
positive and PI positive). As shown in Fig. 5B, G. flavum extract induced the apparition of an apoptotic sub population in MDA-MB-231 and Hs578T cell lines (24.3% compared to 5% in the control at 24 h and 30.8% compared to 3.4% in the control at 48 h for MDA-MB-231). In accordance with DAPI staining, only minimal cell death was observed with MCF10A cells, even after 48 h of treatment, thus confirming the specific effect of G. flavum on cancer cell viability.

**G. flavum extract decreases glioma tumor growth in vivo**

To further confirm the anti-tumoral effect of G. flavum extract in vivo, we used a robust and highly reproducible glioma progression model where U87-MG human glioma cells that are grafted onto the vascularized chicken CAM develop into a tumor within a short period of time (Hagedorn et al. 2005). Tumors were treated daily by a local deposition of G. flavum extract (100 μg/ml) or vehicle from the second to the seventh day post-implantation. At the end of the experiment, treated tumors appeared clearly smaller and visibly less vascularized than the control tumors (Fig. 6A). Indeed, the volume of treated experimental glioma was reduced up to 70% when compared to control tumors (Fig. 6B). The hematoxylin and eosin staining of histological sections generally showed a massive necrosis and infiltration of immune cells in G. flavum treated tumors (Fig. 6C, see inserts). Finally, the selective lectin staining of tumoral vasculature demonstrated noticeable differences between treated and non-treated tumors. Treated tumors consistently showed smaller vessels presenting with reduced lumen when compared with vessels in untreated tumor (Fig. 6D).

**Discussion**

Medicinal herbs and plants continue to play a significant role in drug discovery and development, particularly in cancer research. Previous phytochemical analysis of G. flavum has shown that it is rich in several aporphine alkaloids: glaucine, isocorydine, protopine and isoboldine (Yakhontova et al. 1973). More recent studies reported the presence of other minor alkaloids such as adihydrochelirubine, dihydrosanguinarine, norsanguinarine and dihydrochelerythrine. Several other plants of the genus Papaveraceae are nowadays used to treat human tumors including Chelidonium majus, Sanguinaria canadensis L. and Macleaya cordata (Ahmad et al. 2000; Chmura et al. 2000). To date no study has reported anti-neoplastic activity for G. flavum.

In this study, we present the first evidence that G. flavum alkaloid root extract exerts a tumor cell growth inhibitory activity by using *in vitro* and *in vivo* experimental models. We show that G. flavum...
extract decreased the viability of all breast cancer cell lines analyzed in this study in a dose specific manner, while it did not affect human normal cells including mammary epithelial cells, fibroblasts and endothelial cells. A previous study demonstrated that at low concentrations, sanguinarine strongly inhibited the growth of all tested tumor and normal cell lines. With normal human fibroblasts showing a similar sensitivity to that of cancer cells, and no differential cytotoxicity could be observed (Debiton et al. 2003). In contrast, G. flavum extract exhibited a selective effect on cancer cells suggesting that this effect could be attributed to the major alkaloids of this plant (aporphine alkaloids) rather than to the minor quaternary benzo[c]phenanthridine alkaloids (e.g. sanguinarine). Interestingly, the HPLC profile revealed that the main compound of the root of G. flavum is protopine. It has been recently reported that protopine exhibited an anti-proliferative effect by induction of tubulin polymerization and mitotic arrest on human hormone refractory prostate cancer cells (Chen et al. 2012). This evidence indicates that protopine might be responsible for the anticancer activity of G. flavum dichloromethane extract reported in this study.

Disturbance of the cancer cell cycle is one of the therapeutic targets for development of new anticancer drugs (Carnero 2002). We showed that G. flavum extract induces G2/M arrest on breast cancer cells without affecting the cell cycle distribution in MCF10A cells. We further demonstrated that the anti-proliferative effects of G. flavum extract are linked, at least in part, with the specific induction of p21 expression in breast cancer cells. Cyclin B1 plays an important role in the regulation of G2/M transition. Flow cytometry studies performed on cycling cells reported that the level of cyclin B1 protein accumulates substantially during G2 phase and before cells enter mitosis, peaks during metaphase, and declines rapidly as the cells proceed through anaphase (King et al. 1994; Widrow et al. 1997). We observed an accumulation of cyclin B1 after 12 h while its expression level tended to slightly decrease after 48 h of treatment. As such, cyclin B1 accumulation is a marker of cells stopped in G2 and/or M cell cycle phases.

The process of programmed cell death, or apoptosis is an important homeostatic mechanism that balances cell division and cell death to maintain appropriate cell number in tissues (Elmore 2007).

![Fig. 6. G. flavum extract inhibits glioma tumor growth in vivo using CAM model: (A) representative pictures of 3 control (DMSO) and 3 G. flavum treated (100 μg/ml) experimental glioma tumors grown on CAM (dotted line), (B) tumor volume was calculated as described in “Material and methods” section. G. flavum extract induced a significant decrease of tumor volume compared with the controls. Results are expressed as the mean ± SD of 10 replicates of a representative experiment (n = 3). ***p < 0.001. (C) H&E staining of glioma tumor sections shows massive necrosis with infiltration of immune cells in G. flavum treated tumors that were not generally observed in control tumors, (D) specific FITC-lectin staining was used for visualization of blood vessels in the tumors shown in panel A. Tumor-associated vasculature was visibly less developed in treated tumors when compared to control tumors. Nuclei appeared blue after TOPRO-3 staining (magnification 100x).](http://dx.doi.org/10.1016/j.phymed.2013.06.007)
Disturbance of apoptosis pathways is a common feature of cancer cells and thus represents one of the strategies for anticancer drug development. Our findings demonstrate that G. flavum extract significantly inhibited cell viability through the specific induction of apoptosis in breast cancer cells. An interesting finding in the present study is that alkaloid extract of G. flavum committed cells to apoptosis at a concentration that was below the concentration range reported for other plant extracts (Cheng et al. 2005). Altogether our results indicate that G. flavum treated MDA-MB-231 show an increased p21 expression, are arrested in G2/M and driven to apoptotic death. Further experiments are needed to dissect cell cycle events and all molecular players associated with G. flavum treatment.

Our in vitro findings urged us to test whether G. flavum extract has anti-tumoural effects in vivo. For this purpose, we used a CAM tumor glioma cell model which allows the evaluation of both tumor growth and tumor associated-angiogenesis in vivo. Interestingly, we observed a significant impact of G. flavum treatment on both processes. Treated experimental tumors were significantly smaller and less vascularized, as they appeared whiter than fully vascularized control tumors. These observations suggest for the first time that G. flavum extract possesses not only an anti-proliferative effect on cancer cells but may also affect endothelial cells and impede angiogenesis. Hematoxylin and eosin staining exhibited large zones of necrosis that could be associated with less vascularized regions in treated tumor sections when compared to untreated tumors. In light of these data, it is tempting to speculate that inhibition of tumor growth in vivo by G. flavum is associated with induction of apoptotic processes and/or limited neovessel formation inside the tumor. Ongoing and further studies will help define the potential anti-angiogenic activity of G. flavum.

Conclusion

In summary, we demonstrate for the first time that G. flavum root extract inhibits the growth of breast cancer cells by causing specific cell cycle arrest in G2/M phase and leading to apoptosis, without affecting normal breast cells. These anticancer effects of G. flavum extract could be attributed to the alkaloid protopine, which is the major compound of the root. This hypothesis remains nevertheless to be confirmed. Besides, additional studies are necessary to identify the possible correlation between the anticancer activity and the major alkaloids present in G. flavum extract to ensure the proper medicinal use of this natural wealth, which could lead to the potential development of an effective cancer chemotherapy agent.

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