ARTICLE
Journal of Cellular Biochemistry 113:2957–2966 (2012)

Journal of Cellular Biochemistry

N-Glycan Biosynthesis Inhibitors Induce In Vitro Anticancer Activity in Colorectal Cancer Cells

Julio Cesar Madureira de-Freitas-Junior,[1,2] Lilian Golçalves Bastos,[1] Carlos Alberto Freire-Neto,[1] Bárbara Du Rocher,[3] Eliana Saul Furquim Werneck Abdelhay,[3] and José Andrés Morgado-Díaz[1*]

[1] *Divisão de Biologia Celular, Coordenação de Pesquisa, Instituto Nacional de Câncer, 37André Cavalcanti Street, 5th Floor, CEP: 20230-051, Rio de Janeiro, RJ, Brazil*

[2] *Programa de Pós-Graduação em Biociências, Universidade do Estado do Rio de Janeiro, 87 Vinte e oito de Setembro Avenue, 4th Floor, CEP: 20551-030, Rio de Janeiro, RJ, Brazil*

[3] *Centro de Transplante de Mudula Óssea, Instituto Nacional de Câncer, 23 Cruz Vermelha Square, 7th Floor, CEP: 20230-130, Rio de Janeiro, RJ, Brazil*

ABSTRACT

During malignant transformation, changes in the expression profile of glycans may be involved in a variety of events, including the loss of cell–cell and cell–matrix adhesion, migration, invasion, and evasion of apoptosis. Therefore, modulation of glycan expression with drugs has promising therapeutic potential for various cancer types. In this study, we investigated the in vitro anticancer activity of the *N*-glycan biosynthesis inhibitors (swainsonine and tunicamycin) in cells derived from colorectal cancer (CRC). We also examined whether these inhibitors are able to induce radiosensitization and toxicity when used in combination with cisplatin or irinotecan, two current anticancer drugs. Our results show that treatment with tunicamycin inhibits cellular mechanisms related to the malignant phenotype, such as anchorage-dependent and anchorage-independent colony formation, migration and invasion, in undifferentiated HCT-116 colon cancer cells, whereas swainsonine only inhibits cell migration. We also observed that tunicamycin, but not swainsonine, caused radiosensitivity in HCT-116 cells. Moreover, the combination of swainsonine with cisplatin or irinotecan enhanced their toxicity in HCT-116 cells, while the combination of tunicamycin with these drugs had no effect. Given these results, we suggest that the modulation of *N*-glycan biosynthesis appears to be a potential therapeutic tool for CRC treatment because inhibition of this process induced anticancer activity in vitro. Additionally, the inhibition of the *N*-glycan biosynthesis in combination with chemotherapic drugs is a promising therapeutic strategy for enhancing radiation therapy. J. Cell. Biochem. 113: 2957–2966, 2012.

KEY WORDS: TUNICAMYCIN; RADIOSENSITIVITY; COLORECTAL CANCER; *N*-GLYCOSYLATION

Colorectal cancer (CRC) represents the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 worldwide [Ferlay et al., 2010; Jemal et al., 2011]. In Brazil, the statistics are in accord with global incidence as seen in the recent data from the Instituto Nacional de Câncer [INCA e Ministério da Saúde, Estimativa, 2012]. Various studies have shown that alterations in the PI3K, Wnt, ERK1/2, and PKA pathways and pro-inflammatory signaling are involved in CRC progression [Brand et al., 2006; Chung et al., 2007; Leve et al., 2008; Tanaka et al., 2008; de Araújo et al., 2010]. However, the role of post-translational protein modification in this process remains poorly understood and warrants further investigation. Glycans are carbohydrates covalently bound to proteins or lipids, and modification of their expression—particularly changes in asparagine-linked oligosaccharides—is a critical step for cellular transformation in CRC cells [Murata et al., 2000]. Using cells derived from this cancer type, we previously showed that increased metastatic potential is correlated

Grant sponsor: CNPq; Grant number: 573806/2008-0; Grant sponsor: FAPERJ; Grant number: E26/170.026/2008; Grant sponsor: Ministerio da Saude-Brazil.

*Correspondence to: José Andrés Morgado-Díaz, Divisão de Biologia Celular, Coordenação de Pesquisa, Instituto Nacional de Câncer, 37André Cavalcanti Street, 5th Floor, CEP: 20230-051, Rio de Janeiro, RJ, Brazil. E-mail: jmorgado@inca.gov.br

Manuscript Received: 9 March 2012; Manuscript Accepted: 23 April 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 2 May 2012

DOI 10.1002/jcb.24173 • © 2012 Wiley Periodicals, Inc.

with changes in cell surface glycan expression [de Albuquerque Garcia Redondo et al., 2004].

N-glycan biosynthesis can be blocked using drugs, such as swainsonine or tunicamycin. Swainsonine inhibits the α-mannosidase II enzyme, thereby blocking the formation of complex-type *N*-glycans [Elbein, 1987], and tunicamycin blocks *N*-glycan biosynthesis by inhibiting the formation of a lipid-linked oligosaccharide precursor [Mahoney and Duksin, 1980]. In this context, some studies have used these inhibitors as potential new anticancer therapeutic drugs. For instance, swainsonine has been used as a therapeutic agent in Phase I and II trials [Goss et al., 1997; Maeder, 2002], and treatment with this drug reduces 5-fluorouracil tolerance in the multistage resistance of CRC cell lines [Hamaguchi et al., 2007]. Additionally, other in vitro and in vivo studies showed that swainsonine inhibits the growth of human gastric carcinoma [Sun et al., 2007]. On the other hand, tunicamycin treatment in combination with doxorubicin, epidoxorubicin, vincristine or cisdiamminedichloroplatinum enhances the toxicity effect in multidrug-resistant human ovarian cystadenocarcinoma cells [Hiss et al., 2007]. Tunicamycin also enhances erlotinib-induced cell growth inhibition in non-small cell lung cancer cells [Ling et al., 2009] and radiosensitizes pancreatic adenocarcinoma and glioma cell lines [Contessa et al., 2008]. Recently, we reported that the inhibition of N-linked glycosylation by tunicamycin induces functional E-cadherin-mediated cell–cell adhesion, leading to the development of a differentiated-like phenotype in undifferentiated CRC cells, which supports the use of this drug as a chemotherapeutic agent in models of CRC [de Freitas Junior et al., 2011].

However, while these in vitro and in vivo studies suggest that complex carbohydrates have promising therapeutic potential for various cancers [Contessa et al., 2010; Gerber-Lemaire and Juillerat-Jeanneret, 2010], the effects of *N*-glycan biosynthesis inhibitors on the malignant phenotype of CRC cells remains unclear. Furthermore, it is known that the CRC treatment involves combination with cytotoxic chemotherapeutics, radiotherapy, and biologic agents. However, this treatment appears to have reached a plateau due to severe adverse effects reduces the treatment adhesion, and drug resistance development. Therefore, it is necessary to test novel therapeutic agents and combinations that target specific biological pathways involved with tumor growth and dissemination [Winder and Lenz, 2010].

In the present study, the effects of treatment with *N*-glycan biosynthesis inhibitors alone and in combination with irinotecan and cisplatin on cytotoxicity, anchorage-dependent and anchorage-independent colony formation, migration, invasion, and radiosensitization were examined to understand the mechanisms of their anticancer activity.

MATERIALS AND METHODS

MATERIALS

Mouse monoclonal anti-E-cadherin (36 clone) was obtained from BD Biosciences (San Diego, CA), and mouse monoclonal anti-tubulin was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Peroxidase-conjugated anti-mouse IgG was purchased from Sigma Chemical Co (St. Louis, MO). The fluorescein-conjugated lectins L-PHA (*Phaseolus vulgaris* L–Leukoagglutinating) and E-PHA (*P. vulgaris* E—Erythroagglutinating) were purchased from United States Biological (Swampscott, MA). Tunicamycin (A1 homolog) and swainsonine were obtained from Sigma Chemical Co. Matrigel was purchased from BD Biosciences.

CELL CULTURE

The colon carcinoma-derived cell lines Caco-2, HCT-116, and HT-29 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in DMEM (GIBCO—Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), penicillin G (60 mg/L), and streptomycin (100 mg/L). For experimental purposes, cells were seeded on culture flasks, plates or transwell polycarbonate filters with an 8 μm pore size (Costar, Cambridge, MA), which were either uncoated or coated with Matrigel.

TREATMENT WITH *N*-GLYCOSYLATION INHIBITORS

Tunicamycin and swainsonine stock solutions were diluted in culture medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum to final concentrations of 5.75, 11.5, 23, and 46 μM for swainsonine and 0.3, 0.6, 1.2, or 2.4 μM for tunicamycin. Cells were treated with the drugs for 24, 48, or 72 h and prepared for subsequent analyses.

CELL VIABILITY ANALYSIS

The cells (2×10^3) were seeded and cultured in 96-well plates and treated with the drugs for 24, 48, or 72 h before incubation with MTT (Sigma Chemical Co.). The cells were maintained for 2 h at 37°C and centrifuged at 1,500*g* for 5 min. The supernatant was removed, and the crystals were dissolved in DMSO. The absorbance at 538 nm was measured with a Spectra Max 190 spectrophotometer (Molecular Devices, Sunnyvale, CA).

TOTAL CELL LYSATES AND WESTERN BLOT

Total cell lysates were obtained by incubating the cells in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 2 mM EDTA, 10 mM HEPES (pH 7.4)) containing 20 mM NaF, 1 mM orthovanadate and a protease inhibitor cocktail (1:100 dilution) for 30 min at 4°C. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was removed and stored at −20°C. For Western blot, equal amounts of protein (30 μg/lane) from the total cell lysates were separated on 10% SDS–PAGE and transferred onto nitrocellulose sheets. The membranes were blocked, incubated overnight with primary antibodies to the selected proteins, and then incubated for 1 h with peroxidase-conjugated secondary antibodies. Proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences GE, Buckinghamshire, UK). All blots were stripped and reprobed with an anti-tubulin antibody to ascertain equal protein loading.

ANALYSIS OF LECTIN LABELING BY FLOW CYTOMETRY

Cells were washed, collected from plates, and centrifuged at 1,500*g* for 3 min, and the precipitate was resuspended in 100 μl of PBS. After incubation for 20 min at room temperature with the

FITC (fluorescein isothiocyanate)-conjugated lectins E-PHA (*P. vulgaris* E) or L-PHA (*P. vulgaris* L) at a final concentration of 50 μg/ml, the cells were collected by centrifugation, washed three times with PBS, and fixed in a 4% paraformaldehyde solution. Cells were analyzed by flow cytometry (FASCalibur, Becton–Dickinson, Mountain View, CA), with unstained cells serving as controls. Fluorescence histograms and mean fluorescence data were created and analyzed with the CellQuest software (Becton–Dickinson).

ANCHORAGE-DEPENDENT AND INDEPENDENT COLONY FORMATION

For anchorage-dependent colony formation analysis, a low density of cells (5×10^2) was seeded in 96-well plates using culture media with or without the inhibitors. After 24 or 48 h, the culture medium was removed and substituted with drug-free medium. The cells were grown for 7 days, fixed with ethanol for 10 min, and stained with a crystal violet solution (0.05% crystal violet and 20% methanol). Cells were washed twice with water and then solubilized with methanol. The absorbance at 595 nm was measured with a Spectra Max190 spectrophotometer (Molecular Devices). For anchorage-independent colony formation analysis, the cells were pre-treated with the inhibitors for 24 h and then seeded and cultured in semi-solid media. The cells were growth for 15 days, and the colonies were observed using an Axio Observer.Z1 microscope (Carl Zeiss, Inc., Germany) equipped with an AxioCam HRc and AxioVision Release 8.2 image analyzer.

CELL MIGRATION ASSAY AND CELL INVASION ASSAY

For the cell migration assay, cells (1.5×10^4) were seeded in the upper surface of transwell inserts with an 8 μm pore size (Costar) and allowed to adhere for 4 h. Then, the inhibitors were added to the cell culture medium. After 48 h, the cells on the upper surface were removed and those from the lower surface were fixed with ethanol for 10 min, stained with crystal violet and observed using an Axio Observer.Z1 microscope (Carl Zeiss, Inc.). For the cell invasion assay, a similar strategy was used. However, in this case, the transwell inserts were coated with Matrigel before cell seeding. For quantification, five fields were counted for each assay.

RADIOSENSITIZATION ASSAY

Cells (2×10^3) were seeded in 96-well plates and allowed to adhere for 4 h. Then, the *N*-glycosylation inhibitors were added to the cell culture medium, and after 24 h, the culture medium was removed and substituted with drug-free medium. The cells were immediately irradiated using a 137 Cs Irradiator (IBL 437) with a single dose of 5 Gy at a dose rate of 2.54 Gy/min. A cell viability assay was performed 0, 24, 48, 72, and 96 h after irradiation using the MTT technique.

For combined drug treatments with irradiation, cells (2×10^3) were seeded in 96-well plates for 4 h. Then, the cells were pre-treated with tunicamycin or swainsonine, and after 24 h, the culture medium was removed and substituted with drug-free medium or cisplatin/irinotecan-containing-medium. Immediately after this substitution, the cells were irradiated as previously indicated. Cell viability was assessed 96 h after irradiation using the MTT assay.

STATISTICAL ANALYSIS

One-way ANOVA was performed with the GraphPad Prism 4.02 software (GraphPad Software, Inc., San Diego, CA). We considered data from three independent experiments to be statistically significant at $P < 0.05$. Graphical data are presented as the means ± SEM.

RESULTS

INHIBITION OF *N*-LINKED GLYCOSYLATION DIFFERENTIALLY AFFECTS THE CELL VIABILITY OF COLON ADENOCARCINOMA CELLS

To determine the cytotoxic effects of *N*-glycan biosynthesis inhibitors, cells were treated with 5.75, 11.5, 23, and 46 μM of swainsonine or 0.3, 0.6, 1.2, and 2.4 μM of tunicamycin, and the cell viability was analyzed after 24, 48, and 72 h using the MTT assay. We observed no significant changes in cells treated with swainsonine (Fig. 1A), but a differential reduction of cell viability in a time- and dose-dependent manner was observed in Caco-2, HCT-116, and HT-29 cells treated with tunicamycin (Fig. 1B). Caco-2 was found to be the most resistant cell line because only the highest concentration (2.4 μM) of tunicamycin induced a significant decrease in cell viability at 72 h ($P < 0.01$). In contrast, HT-29 was the most sensitive cell line, given that even the lowest concentration (0.3 μM) of tunicamycin induced a significant decrease in cell viability at 24 h ($P < 0.05$). HCT-116 cells displayed an intermediate sensitivity rate compared to Caco-2 and HT-29; a concentration lower than 2.4 μM significantly affected the cell viability after only 72 h of treatment. Together, these data show that inhibition of N-linked glycosylation differentially affects the viability of cell lines derived from colon adenocarcinoma. On the basis of these results, we chose HCT-116 cells, which showed an intermediate sensitivity rate to tunicamycin, for subsequent functional analyses and always conducted these analyses using treatment conditions that did not affect HCT-116 cell viability.

LOW DOSES OF TUNICAMYCIN PARTIALLY INHIBIT N-LINKED GLYCOSYLATION WITHOUT AFFECTING CELL VIABILITY

To functionally validate the pharmacological inhibition of N-linked glycosylation by swainsonine and tunicamycin, we first analyzed the gel mobility shift of E-cadherin, a glycoprotein known to be *N*-glycosylated [Pinho et al., 2009]. After 24 and 48 h of treatment of HCT-116 cells with swainsonine, no change in the gel mobility of E-cadherin was observed. However, a band with lower molecular weight, indicating a shift in E-cadherin mobility, was observed after tunicamycin treatment; the constitutively *N*-glycosylated form can also be visualized (Fig. 2A). This result demonstrates that even low doses of tunicamycin are able to partially inhibit N-linked glycosylation without affecting HCT-116 cell viability. To further confirm these findings, we performed a lectin-labeling assay using flow cytometry using the lectins E-PHA and L-PHA, which are specific for bisected and branched complex-type *N*-glycans, respectively [Granovsky et al., 2000; Iijima et al., 2006]. We observed that treatment with swainsonine and tunicamycin, at all concentrations and times used, produced detectable inhibition of complex-type *N*-glycan biosynthesis in HCT-116 cells (Fig. 2B). This apparent contradiction regarding the above result observed for the swainsonine treatment may be explained by the inhibition of downstream *N*-glycan biosynthesis caused by this

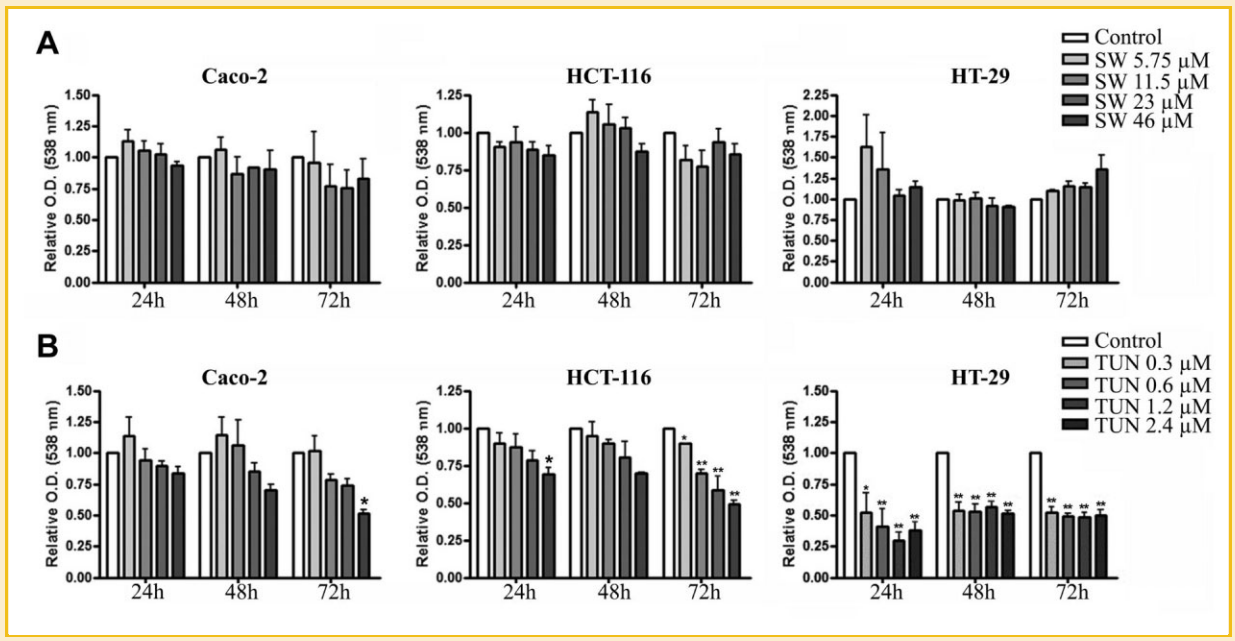

Fig. 1. Effects of swainsonine and tunicamycin on Caco-2, HCT-116, and HT-29 cell viability. The bar graphs show the relative optical density (538 nm) after 24, 48, and 72 h of treatment with swainsonine (A) or tunicamycin (B). At all times analyzed the values were normalized to the control group at each time point. Error bars indicate the means ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA.

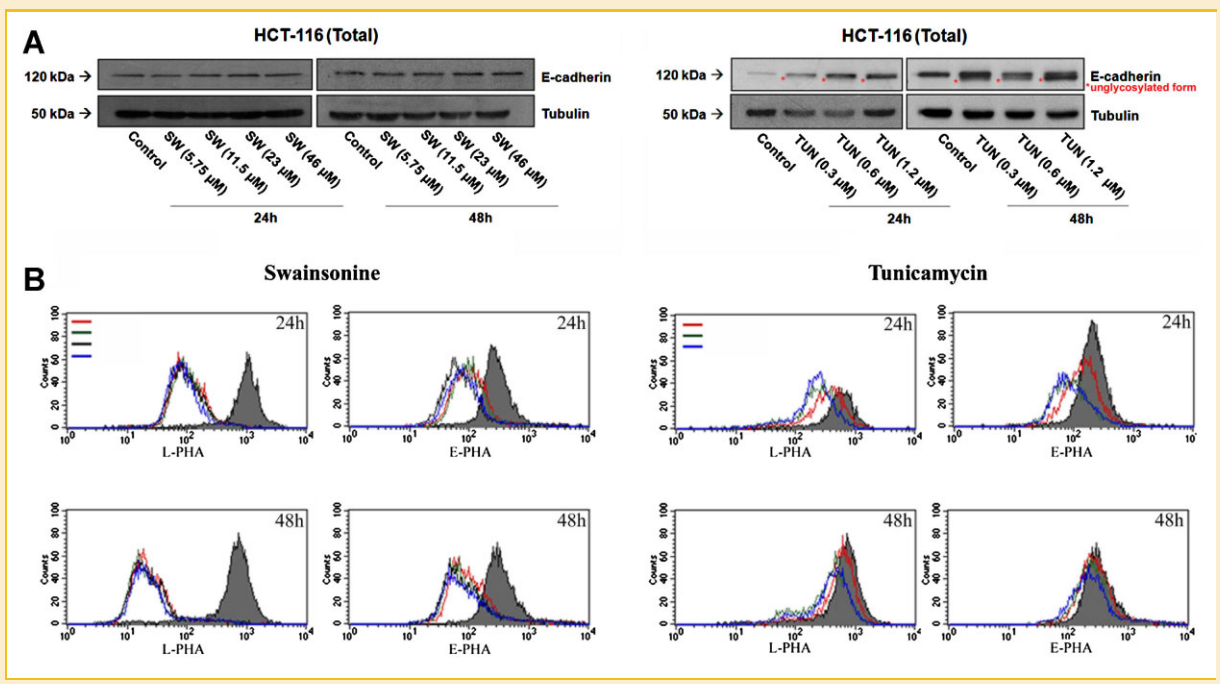

Fig. 2. A: Effects of swainsonine and tunicamycin on the gel shift mobility of the E-cadherin glycoprotein in HCT-116 cells. After drug treatment, total cell lysates were obtained and analyzed by Western blot for E-cadherin. Asterisks indicate the unglycosylated form of E-cadherin. Tubulin was used as a loading control. B: Effects of swainsonine and tunicamycin on the expression of complex-type *N*-glycans in HCT-116 cells. After drug treatment, the cells were incubated with FITC-conjugated E-PHA or L-PHA lectins. Labeling on the cell surface was analyzed by flow cytometry, and fluorescence histograms were created with the Cell Quest software. Unstained cells were used as controls. Shifts toward the left on the histograms indicate lower fluorescence intensity. Histograms colors: for swainsonine, control (gray), 5.75 μM (red), 11.5 μM (green), 23 μM (blue), and 46 μM (black); for tunicamycin, control (gray), 0.3 μM (red), 0.6 μM (green), and 1.2 μM (blue). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

drug, which might have produced an undetectable shift in E-cadherin mobility on a mini-gel, compared with tunicamycin.

INHIBITION OF N-LINKED GLYCOSYLATION BY TUNICAMYCIN SUPPRESSES THE MIGRATION AND INVASION OF HCT 116 CELLS

Cancer cell migration and invasiveness are considered crucial steps in the metastatic cascade [Lin et al., 2005]. Therefore, we examined the status of these events in the presence of various concentrations of the inhibitors. We observed that the concentrations of swainsonine used in this study (5.75, 11.5, 23, and 46 μM) were able to significantly suppress cell migration, whereas they did not produce any significant changes in the invasion of HCT-116 cells in relation to the control cells (Fig. 3A,B). Moreover, 0.3, 0.6, and 1.2 μM of tunicamycin significantly suppressed cell migration,

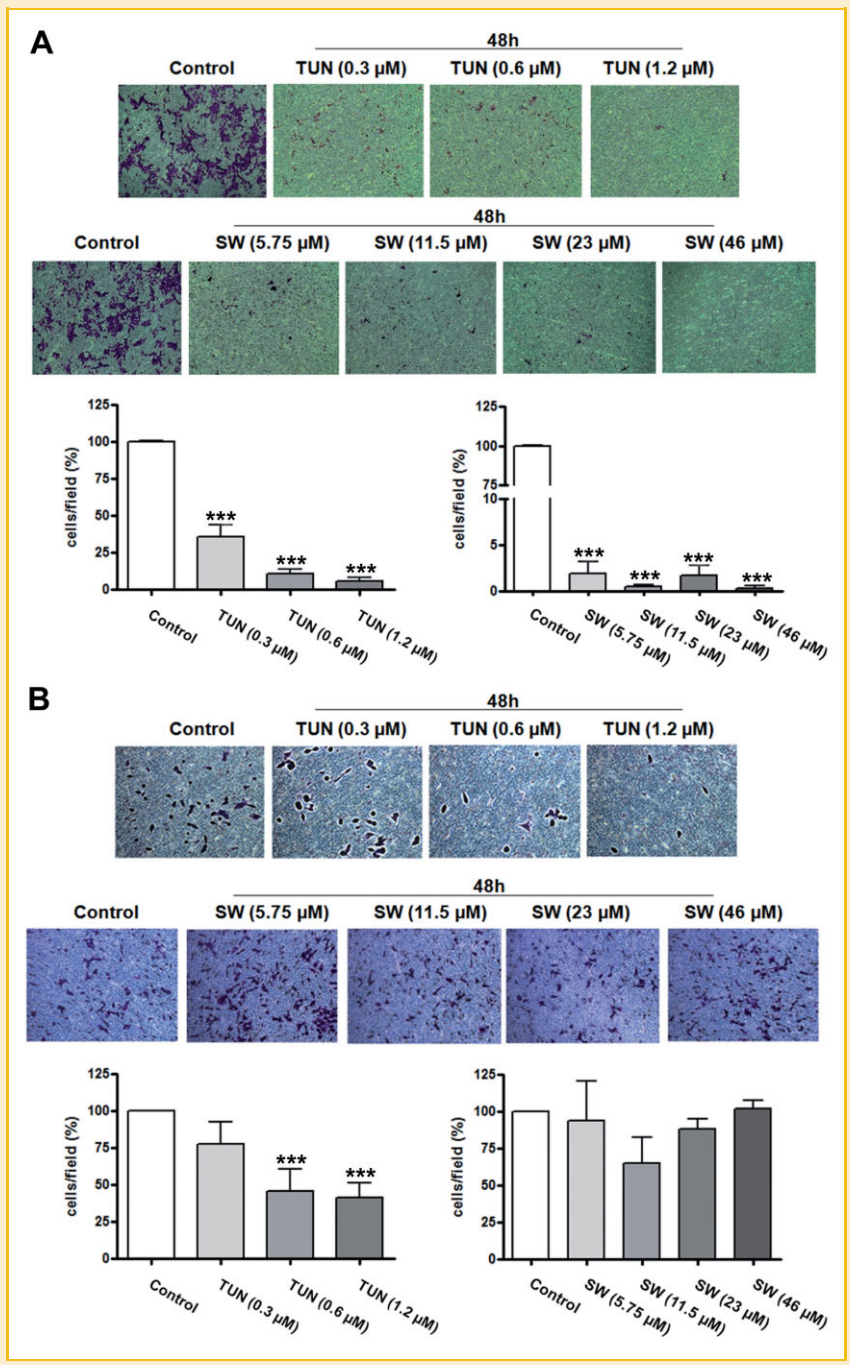

Fig. 3. Effects of swainsonine and tunicamycin on HCT-116 cell migration and invasion in vitro. A: Representative images of cell migrating through the 8 μm pore of a polycarbonate membrane. Cells were stained with crystal violet. B: Representative images of cell invasion through Matrigel and the 8 μm pore of a polycarbonate membrane. Cells were stained with crystal violet. The bar graphs show the amount of cells/field. Error bars indicate the means ± SEM (n = 3). $^{*}P < 0.05$, ANOVA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

while concentrations of 0.6 and 1.2 μM significantly inhibited the invasion of HCT-116 cells (Fig. 3A,B). Collectively, these data strongly suggest that inhibition of *N*-glycosylation suppresses crucial features related to the malignant cancer phenotype. However, downstream inhibition using swainsonine did not alter HCT-116 cell invasion.

TREATMENT WITH TUNICAMYCIN INHIBITS ANCHORAGE-DEPENDENT AND ANCHORAGE-INDEPENDENT COLONY FORMATION IN HCT-116 CELLS

Because the abilities of tumor cells to form anchorage-dependent colonies from a single cell and to form colonies independent of anchorage are important features of metastatic potential [Quintana et al., 2008], we evaluated the effects of *N*-glycan biosynthesis inhibitors in this context. Figure 4A,B shows that none of the concentrations of swainsonine used in this study produced significant changes in these two parameters in HCT-116 cells compared with the control group. Nevertheless, after 24 and 48 h of treatment with different concentrations of tunicamycin (0.3, 0.6, and 1.2 μM), a significant inhibition of anchorage-dependent colony formation was observed in these cells ($P < 0.01$). Furthermore, pre-treatment with tunicamycin for 24 h also induced a significant suppression of anchorage-independent growth, which was enhanced with higher doses ($P < 0.05$ for 0.3 μM and $P < 0.01$

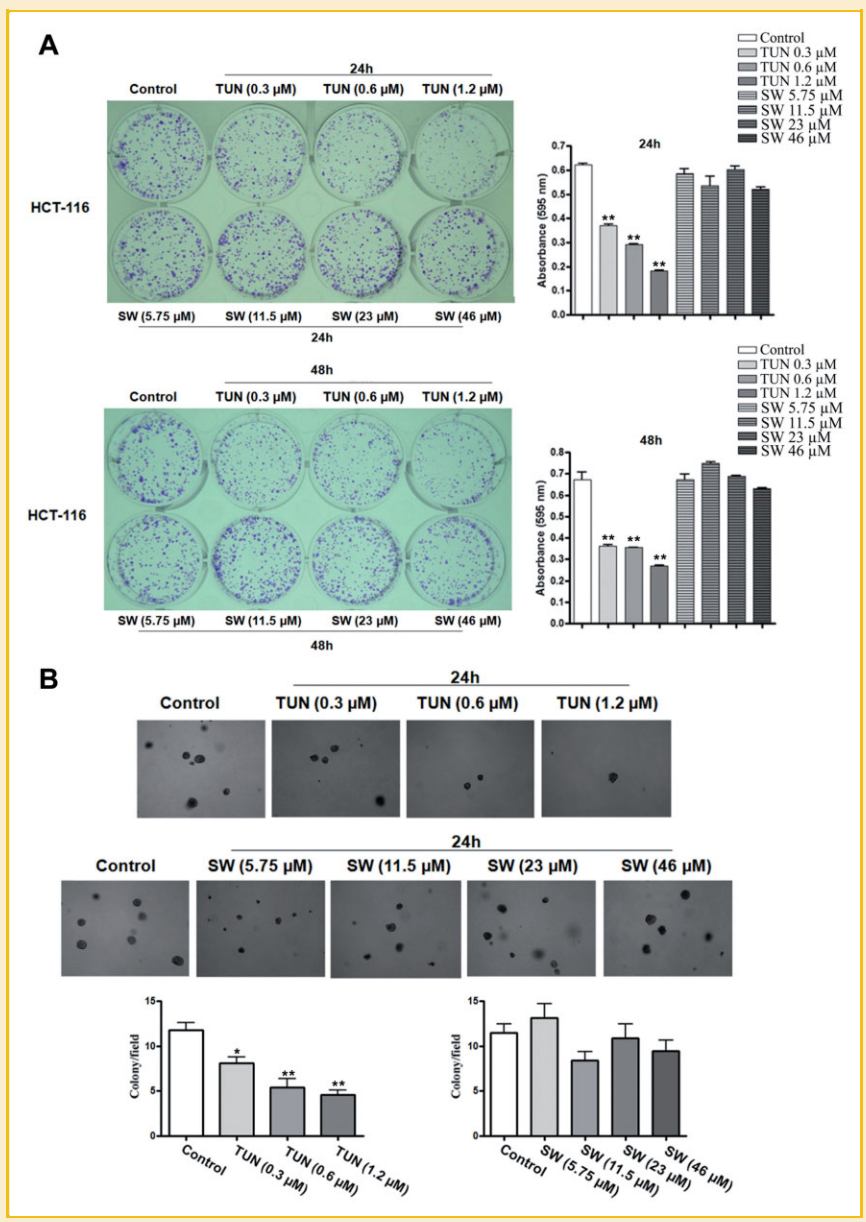

Fig. 4. Effects of swainsonine and tunicamycin on HCT-116 anchorage-dependent and anchorage-independent colony formation. A: Representative photographs of anchorage-dependent colonies stained with crystal violet. The bar graphs show the optical density (595 nm) after staining. B: Representative images of unstained anchorage-independent colonies obtained by phase contrast microscopy. The bar graphs show the number of colonies/field. Error bars indicate the means + S.E.M (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

for 0.6–1.2 μM) (Fig. 4B). Together, these data show that inhibition of N-linked glycosylation by tunicamycin blocks the establishment of anchorage-dependent colonies from a single cell at the same level as growth-independent anchorage.

INHIBITION OF N-LINKED GLYCOSYLATION BY TUNICAMYCIN PROMOTES RADIOSENSITIVITY IN HCT-116 CELLS

In view of the relevance of radiotherapy to CRC treatment [Glynne-Jones and Kronfli, 2011], we also evaluated whether the *N*-glycosylation inhibitors are able to induce radiosensitivity in HCT-116 cells. We observed that when cells were treated with the inhibitors prior to irradiation, both swainsonine (23 μM) and tunicamycin (1.2 μM), at concentrations which did not interfere with cell viability, induced radiosensitivity after 96 h of pre-treatment, but this induction was only statistically significant ($P < 0.01$) with tunicamycin treatment (Fig. 5A). These results were encouraging because no other published data has shown the radiosensitization potential of *N*-glycan biosynthesis inhibitors in cells derived from CRC.

DOWNSTREAM INHIBITION OF N-GLYCAN BIOSYNTHESIS BY SWAINSONINE ENHANCES IRINOTECAN/CISPLATIN-INDUCED CYTOTOXICITY IN IRRADIATED CELLS

Acquisition of chemoresistance reduces drug effectiveness and represents a major problem in cancer chemotherapy [Hiss et al., 2007]. Therefore, we tested whether the combination of *N*-glycan biosynthesis inhibitors with classic anticancer drugs, such as irinotecan, an inhibitor of topoisomerase 1 and cisplatin, that promote DNA damage could show an increase in their cytotoxic effects in irradiated cells. First, we evaluated the sensitivity of HCT-116 to irinotecan and cisplatin treatment and observed that irinotecan induced a cytotoxicity higher than cisplatin (Fig. 5B). We then further analyzed the cytotoxic effects of these drugs in cells pre-treated with *N*-glycosylation inhibitors under radiation conditions. We observed that tunicamycin did not enhance irinotecan/cisplatin-induced cytotoxicity in irradiated cells, but swainsonine caused a significant irinotecan/cisplatin-induced cytotoxicity under these condition (Fig. 5C). These data suggest that downstream inhibition of *N*-glycan biosynthesis with swainsonine in combination with irinotecan or cisplatin might be a useful therapeutic strategy in CRC cells.

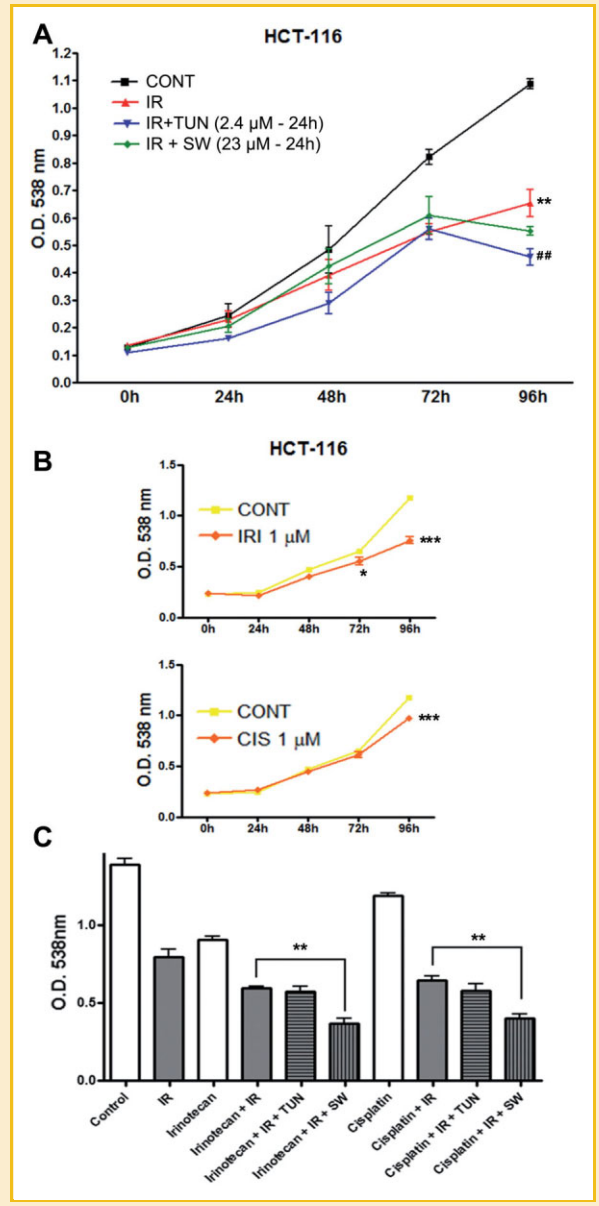

Fig. 5. A: Effects of swainsonine and tunicamycin on HCT-116 radiosensitization. After pre-treatment (24 h) with inhibitors, cells were irradiated (5 Gy). Cell viability was evaluated after 0, 24, 48, 72 and 96 h. The line graph shows the amount of optical density (538 nm). B,C: Effects of swainsonine and tunicamycin on irinotecan/cisplatin-induced cytotoxicity in irradiated cells. The cytotoxic effects of irinotecan (IR) and cisplatin (CIS) were evaluated over 96 h. The line graphs show the optical density (538 nm). After pre-treatment (24 h) with *N*-glycan biosynthesis inhibitors, cells were irradiated (5 Gy) and treated with irinotecan or cisplatin; cell viability was evaluated after 96 h. The bar graphs show the relative optical density (538 nm). Error bars indicate the means ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$, ANOVA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

Aberrant glycosylation constitutes a hallmark of epithelial tumor progression [Granovsky et al., 2000; Nita-Lazar et al., 2010]. However, cellular and molecular mechanisms involved in *N*-glycan expression alterations during CRC progression remain to be defined. Furthermore, it is not yet clear whether *N*-glycan biosynthesis could be considered a new target for CRC therapy.

In this study, we initially evaluated the cytotoxic effects of *N*-glycan biosynthesis inhibitors using various CRC cell lines. Our results show that swainsonine did not affect the viability of these cells. Conversely, some studies suggest the inhibition of α-mannosidase II with this drug as a potential antineoplastic alternative in other cell models [Hamaguchi et al., 2007; Sun et al., 2007]. On the contrary, tunicamycin induced a differential reduction in cell viability in a time- and dose-dependent manner in the three cell lines used in this study. A possible explanation for this differential reduction could be that these cells display different

phenotypes and mutational states: Caco-2 is a differentiated cell line with a *P53* mutation; HCT-116 is undifferentiated with a *RAS* mutation, and HT-29 is differentiated with both *RAS* and *P53* mutations, which confer a high proliferation ability [Chantret et al., 1988]. Actually, it is known that the junctional complex plays an important role in the differentiation status of tumor epithelial cells, and any alteration of components of this complex can led to loss of their function. In this context, using these same CRC cell lines as well these inhibitors, we recently demonstrate that altered *N*-glycan expression plays an important role in the loss of adherents junctions stability in undifferentiated CRC cells and that this loss may be associated with the progression of CRC [de Freitas Junior et al., 2011]. Thus, the partial inhibition of the *N*-glycan biosynthesis, may explain the effects reported in the present article. Additionally, there is some evidence that the Ras/Raf pathway could be involved in changes in the pattern of *N*-glycan expression [Lau and Dennis, 2008], but whether the inhibitors used here can modulate this pathway in our model remains to be demonstrated. Our results show for the first time that inhibition of N-linked glycosylation differentially modulates the viability of cells derived from CRC and depends on the differentiation and mutational status of these cells.

To validate whether the drug concentrations and times used in this study produce detectable effects on the inhibition of *N*-glycan biosynthesis without causing deleterious effects on cell viability, we functionally evaluated *N*-glycan expression after treatment with these drugs. We observed that both drugs at the times and concentrations used were able to inhibit *N*-glycosylation in HCT-116 cells, as observed by the gel mobility shift of E-cadherin and the lectin-labeling assay performed using flow cytometry (Fig. 2A,B). The inhibition of *N*-glycosylation with 0.1–0.5 μg/ml tunicamycin disrupts receptor tyrosine kinase signaling in pancreatic adenocarcinoma and glioma cell lines [Contessa et al., 2008]. Moreover, partial inhibition of the *DPAGT1* gene (which encodes the dolichol-P-dependent *N*-acetylglucosamine-1-phosphate transferase) using siRNA induced an *N*-glycosylation blockage without causing deleterious effects on the viability of MDCK cells [Nita-Lazar et al., 2010]. These studies support our finding that the inhibitors used in this study induced the inhibition of *N*-glycosylation without causing deleterious effects on cell viability.

The metastatic potential of malignant cells involves increased cell motility and invasiveness. Thus, to evaluate the in vitro anticancer activity resulting from the inhibition of *N*-glycan biosynthesis, we treated HCT-116 cells with tunicamycin and swainsonine and observed that both drugs inhibited cell migration, whereas only tunicamycin inhibited cell invasion (Fig. 3A,B). The loss of cell–cell and cell–matrix adhesion, which is involved with migratory potential, has been shown to be associated with changes in complex-type *N*-glycan expression in breast cancer cells [Lagana et al., 2006; Pinho et al., 2009]. In fact, the biological functions of cadherins and integrins can be modified by the presence of different glycan patterns on these molecules [Lagana et al., 2006; Pinho et al., 2009]. Curiously, no inhibitory effects on cell invasion were observed after treatment with swainsonine, which also blocks complex-type *N*-glycans. Previous studies have shown that this drug induces, for example, tight cell–cell adhesion in differentiated MDCK cells [Vagin et al., 2008], suggesting that particularly in undifferentiated HCT-116 cells, complex-type *N*-glycans may not be the only *N*-glycans involved in controlling cell invasion; high mannose and hybrid *N*-glycans might also be involved. It is also important to note our previous results showing that tunicamycin induces E-cadherin-mediated cell–cell adhesion in HCT-116 cells, but no effects were observed after treatment with swainsonine (23–46 μM) [de Freitas Junior et al., 2011]. Metastatic potential is also determined by anchorage-dependent and anchorage-independent colony formation, which was also assessed after inhibitor treatment. No inhibitory effect was observed with swainsonine treatment, while tunicamycin promoted inhibition of both types of colony formation. This is the first report to show that the inhibition of *N*-glycan biosynthesis blocks these two types of colony formation in CRC cells, suggesting that *N*-glycan expression is associated with the important characteristic of high metastatic potential in CRC cells.

Although surgery is the primary treatment of CRC, chemoradiation, as a preoperative or post-operative adjuvant therapy, has been explored to improving local control and survival of locally advanced rectal cancer [O'Connell et al., 1994]. Various chemotherapeutic and biologic agents have been used as radiosensitizers in combination with radiotherapy including 5-fluorouracil [Minsky et al., 1997]. In addition, newer chemotherapeutic agents, such as capecitabine, irinotecan, and oxaliplatin, have been introduced as radiosensitizers [Freyer et al., 2001; Kim et al., 2002; Gerard et al., 2003; Mehta et al., 2003]. To date, new therapies, such as those targeting VEGF and EGFR, have contributed to the CRC treatment protocol, but only subgroups of patients benefit as some tumors have developed drug resistance. Therefore, novel chemotherapeutic combinations for the treatment of this cancer type must be examined [Leve and Morgado-Díaz, 2012].

Because in vivo and in vitro evidence has supported the idea that the inhibition of *N*-glycosylation could radiosensitize tumor cells [Contessa et al., 2008, 2010], we tested this hypothesis in our model. We verified that both tunicamycin and swainsonine caused radiosensitivity, but only the effect of tunicamycin treatment was statistically significant. A presumable implication of *N*-glycosylation suppression is an effect on P-glycoprotein (Pgp) maturation [Loo and Clarke, 1999; Contessa et al., 2008]. Because Pgp is an ATP-dependent efflux pump that prevents the intracellular accumulation of cytotoxic anticancer drugs, we tested the combination of classic anticancer drugs used in CRC patients [Nakata et al., 2007; Goodwin and Asmis, 2009], cisplatin or irinotecan, with *N*-glycosylation inhibitors to achieve enhanced cytotoxicity. Swainsonine was able to enhance cisplatin/irinotecan cytotoxicity, but interestingly, no effect was observed when cells were treated in combination with tunicamycin. In human head-and-neck carcinoma cells, different results have been observed, as tunicamycin was demonstrated to enhance sensitivity to cisplatin [Noda et al., 1999], showing that these effects could be tissue/cell-specific. Additionally, it is known that *N*-glycosylation blockade disturbs the protein folding in the endoplasmic reticulum (ER), resulting in ER stress. The adverse effects of accumulating unfolded proteins activate a set of signaling pathways termed the unfolded protein response (UPR). Signaling initiated from the UPR actively participates in autophagy and both intrinsic and extrinsic

apoptosis pathways and under acute prolonged ER stress, the apoptosis is triggered [Smith and Deshmukh, 2007; Clarke et al., 2012]. Further studies are required to understand the molecular details by which N-linked glycosylation increase the radiosensitivity in CRC cells. As previously reported, while tunicamycin is not a potential therapeutic agent due to its narrow efficacy window, a compound with similar biological effects but with a broader therapeutic window could be useful, for example, for cell radiosensitization [Contessa et al., 2010].

In conclusion, we report that blocking *N*-glycan biosynthesis inhibits cellular mechanisms related to the malignant cancer phenotype of undifferentiated CRC cells, specifically HCT-116 cells. Moreover, tunicamycin alone induced HCT-116 cell radiosensitivity, and a combination of swainsonine with cisplatin or irinotecan enhanced the cytotoxic effect in these cells. Finally, these results suggest *N*-glycan biosynthesis as a potential therapeutic target for CRC because the inhibition of *N*-glycan expression influences several aspects of tumor progression.

ACKNOWLEDGMENTS

We are grateful to Dr. Fernando Menton from the Clinical Research Division-INCa for his suggestion in the present study. This study was sponsored by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Ministerio da Saúde—Brasil, and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado de Rio de Janeiro (FAPERJ). This text was reviewed by American Journal Experts.

REFERENCES

Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, Göke B, Dambacher J. 2006. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. J Cell Biochem 97:709–723.

Chantret I, Barbat A, Dussaulx E, Brattain MG, Zweibaum A. 1988. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: A survey of twenty cell lines. Cancer Res 48:1936–1942.

Chung GG, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL. 2007. Tissue microarray analysis of beta-catenin in colorectal cancer shows nuclear phospho-beta-catenin is associated with a better prognosis. Clin Cancer Res 7:4013–4020.

Clarke R, Cook KL, Hu R, Facey CO, Tavassoly I, Schwartz JL, Baumann WT, Tyson JJ, Xuan J, Wang Y, Wärri A, Shajahan AN. 2012. Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. Cancer Res 72:1321–1331.

Contessa JN, Bhojani MS, Freeze HH, Rehemtulla A, Lawrence TS. 2008. Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signaling in tumor cells. Cancer Res 68:3803–3809.

Contessa JN, Mahaveer SB, Freeze HH, Ross BD, Rehemtulla A, Lawrence TS. 2010. Molecular imaging of N-linked glycosylation suggests glycan biosynthesis is a novel target for cancer therapy. Clin Cancer Res 16:3205–3214.

de Albuquerque Garcia Redondo P, Nakamura CV, de Souza W, Morgado-Diaz JA. 2004. Differential expression of sialic acid and N-acetylgalactosamine residues on the cell surface of intestinal epithelial cells according to normal or metastatic potential. J Histochem Cytochem 52:629–640.

de Araújo WM, Vidal FC, de Souza WF, de Freitas Junior JC, de Souza W, Morgado-Diaz JA. 2010. PI3K/Akt and GSK-3β prevents in a differential fashion the malignant phenotype of colorectal cancer cells. J Cancer Res Clin Oncol 136:1773–1782.

de Freitas Junior JC, Du Rocher B, de Souza WF, de Araújo WM, Abdelhay ES, Morgado-Díaz JA. 2011. Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell–cell adhesion and inhibits cell proliferation in undifferentiated human colon cancer cells. Cancer Chemother Pharmacol 68:227–238.

Elbein AD. 1987. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chain. Annu Rev Biochem 56:497–534.

Ferlay J, Shin HR, Bray F, Forman D, Mathers CD, Parkin D. 2010. D. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10. Lyon, France: International Agency for Research on Cancer; Year. Available at: http://globocan.iarc.fr.

Freyer G, Bossard N, Romestaing P, Mornex F, Chapet O, Trillet-Lenoir V, Gérard JP. 2001. Addition of oxaliplatin to continuous fluorouracil, l-folinic acid, and concomitant radiotherapy in rectal cancer: The Lyon R 97-03 phase I trial. J Clin Oncol 19:2433–2438.

Gerard JP, Chapet O, Nemoz C, Romestaing P, Mornex F, Coquard R, Barbet N, Atlan D, Adeleine P, Freyer G. 2003. Preoperative concurrent chemoradiotherapy in locally advanced rectal cancer with high-dose radiation and oxaliplatin-containing regimen: The Lyon R 0-04 phase II trial. J Clin Oncol 21:1119–1124.

Gerber-Lemaire S, Juillerat-Jeanneret L. 2010. Studies toward new anticancer strategies based on alpha-mannosidase inhibition. Chimia (Aarau) 64:634–639.

Glynne-Jones R, Kronfli M. 2011. Locally advanced rectal cancer: A comparison of management strategies. Drugs 71:1153–1177.

Goodwin RA, Asmis TR. 2009. Overview of systemic therapy for colorectal cancer. Clin Colon Rectal Surg 22:251–256.

Goss PE, Reid CL, Bailey D, Dennis JW. 1997. Phase IB clinical trial of the oligosaccharide processing inhibitor swainsonine in patients with advances malignancies. Clin Cancer Res 3:1077–1086.

Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW. 2000. Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat Med 6:306–312.

Hamaguchi J, Nakagawa H, Takahashi M, Kudo T, Kamiyama N, Sun B, Oshima T, Sato Y, Deguchi K, Todo S. 2007. Nishimura, swainsonine reduces 5-fluorouracil tolerance in the multistage resistance of colorectal cancer cell lines. Mol Cancer 6:58.

Hiss DC, Gabriels GA, Folb PI. 2007. Combination of tunicamycin with anticancer drugs synergistically enhances their toxicity in multidrug-resistant human ovarian cystadenocarcinoma cells. Cancer Cell Int 7:5.

Iijima J, Zhao Y, Isaji T, Kameyama A, Nakaya S, Wang X, Ihara H, Cheng X, Nakagawa T, Miyoshi E, Kondo A, Narimatsu H, Taniguchi N, Gu J. 2006. Cell–cell interaction-dependent regulation of N-acetylglucosaminyltransferase III and the bisected *N*-glycans in GE11 epithelial cells. J Biol Chem 281:13038–13046.

Instituto Nacional de Câncer. 2011. Incidência de câncer no Brasil, Instituto Nacional de Câncer Estimativa 2012, Rio de Janeiro.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. Ca Cancer J Clin 61:69–90.

Kim JS, Kim JS, Cho MJ, Song KS, Yoon WH. 2002. Preoperative chemoradiation using oral capecitabine in locally advanced rectal cancer. Int J Radiat Oncol Biol Phys 54:403–408.

Lagana A, Goetz JG, Cheung P, Raz A, Dennis JW, Nabi IR. 2006. Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. Mol Cell Biol 26:3181–3193.

Lau K, Dennis JW. 2008. N-glycans in cancer progression. Glycobiology 18:750–760.

Leve F, Morgado-Díaz JA. 2012. Rho GTPase signaling in the development of colorectal cancer. J Cell Biochem (in press). DOI: 10.1002/jcb.24153

Leve F, de Souza W, Morgado-Díaz JA. 2008. Across-link between protein kinase A and Rho-family GTPases signaling mediates cell–cell adhesion and actin cytoskeleton organization in epitelial cancer cells. J Pharmacol Exp Ther 327:777–788.

Lin M, DiVito MM, Merajver SD, Boyanapalli M, van Golen KL. 2005. Regulation of pancreatic cancer cell migration and invasion by RhoC GTPase and caveolin-1. Mol Cancer 4:21.

Ling YH, Li T, Perez-Soler R, Haigentz M Jr. 2009. Activation of ER stress and inhibition of EGFR N-glycosylation by tunicamycin enhances susceptibility of human non-small cell lung cancer cells to erlotinib. Cancer Chemother Pharmacol 64:539–548.

Loo T, Clarke D. 1999. The human multidrugresistance P-glycoproteinis inactive when its maturation is inhibited: Potential for a role in cancer chemotherapy. FASEB J 13:1724–1732.

Maeder T. 2002. Sweet medicines. Sci Am 287:40–47.

Mahoney WC, Duksin D. 1980. Separation of tunicamycin homologues by reversed-phase high-performance liquid chromatography. J Chromatogr Sci 198:506–510.

Mehta VK, Cho C, Ford JM, Jambalos C, Poen J, Koong A, Lin A, Bastidas JA, Young H, Dunphy EP, Fisher G. 2003. Phase II trial of preoperative 3D conformal radiotherapy, protracted venous infusion 5-fluorouracil, and weekly CPT-11, followed by surgery for ultrasound-staged T3 rectal cancer. Int J Radiat Oncol Biol Phys 55:132–137.

Minsky BD, Cohen AM, Enker WE, Saltz L, Guillem JG, Paty PB, Kelsen DP, Kemeny N, Ilson D, Bass J, Conti J. 1997. Preoperative 5-FU, low-dose leucovorin, and radiation therapy for locally advanced and unresectable rectal cancer. Int J Radiat Oncol Biol Phys 37:289–295.

Murata K, Miyoshi E, Kameyama M, Ishikawa O, Kabuto T, Sasaki Y, Hiratsuka M, Ohigashi H, Ishiguro S, Ito S, Honda H, Takemura F, Taniguchi N, Imaoka S. 2000. Expression of N-acetylglucosaminyltransferase V in colorectal cancer correlates with metastasis and poor prognosis. Clin Cancer Res 6:1772–1777.

Nakata B, Sowa M, Tsuji A, Kamano T, Sasaki K, Fukunaga Y, Takahashi M, Tsujitani S, Mikami Y, Mitachi Y, Nishimura S, Araki H, Yamamitsu S, Hirakawa K, Tominaga S, Shirasaka T, Inokuchi K. 2007. Continuous infusion of 5-fluorouracil with versus without low-dose, consecutive administration of cisplatin in advanced colorectal cancer. A prospective randomized phase II study. J Exp Clin Cancer Res 26:51–60.

Nita-Lazar M, Rebustini I, Walker J, Kukuruzinska MA. 2010. Hypoglycosylated E-cadherin promotes the assembly of tight junctions through the recruitment of PP2A to adherens junctions. Exp Cell Res 316:1871–1884.

Noda I, Fujieda S, Seki M, Tanaka M, Sunaga H, Ohtsubo T, Tsuzuki H, Fan GK, Saito H. 1999. Inhibition of N-linked glycosylation by tunicamycin enhances sensitivity to cisplatin in human head-and-neck carcinoma cells. Int J Cancer 80:279–284.

O'Connell MJ, Martenson JA, Wieand HS, Krook JE, Macdonald JS, Haller DG, Mayer RJ, Gunderson LL, Rich TA. 1994. Improving adjuvant therapy for rectal cancer by combining protractedinfusion fluorouracil with radiation therapy after curative surgery. N Engl J Med 331:502–507.

Pinho SS, Reis CA, Paredes J, Magalhães AM, Ferreira AC, Figueiredo J, Xiaogang W, Carneiro F, Gärtner F, Seruca F. 2009. The role of *N*-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin. Hum Mol Genet 18:2599–2608.

Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson tm, Morrison SJ. 2008. Efficient tumour formation by single human melanoma cells. Nature 456:593–598.

Sun JY, Zhu MZ, Wang SW, Miao S, Xie YH, Wang JB. 2007. Inhibition of the growth of human gastric carcinoma in vivo and in vitro by swainsonine. Phytomedicine 14:353–359.

Smith MI, Deshmukh M. 2007. Endoplasmic reticulum stress-induced apoptosis requires bax for commitment and Apaf-1 for execution in primary neurons. Cell Death Differ 14:1011–1019.

Tanaka MN, Diaz BL, de Souza W, Morgado-Díaz JA. 2008. Prostaglandin E2-EPI and EP2 receptor signaling promotes apical junctional complex disassembly of Caco-2 human colorectal cancer cells. BMC Cell Biol 9:63.

Vagin O, Tokhtaeva E, Iskandar Y, Eugenia S, Sachs G. 2008. Inverse correlation between the extent of *N*-glycan branching and intercellular adhesion in epithelia. J Biol Chem 283:2192–2202.

Winder T, Lenz HJ. 2010. Vascular endothelial growth factor and epidermal growth factor signaling pathways as therapeutic targets for colorectal cancer. Gastroenterology 138:2163–2176.