Published in final edited form as:

Mol Cancer Ther. 2008 October ; 7(10): 3237–3246. doi:10.1158/1535-7163.MCT-08-0398.

FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells

Silvia Fernández de Mattos^{1,#}, Priam Villalonga¹, Jon Clardy², and Eric W-F Lam³

¹ Cancer Cell Biology Group. Institut Universitari d'Investigació en Ciències de la Salut (IUNICS). Departament de Biologia Fonamental. Universitat de les Illes Balears. Crta Valldemossa km 7.5. E-07122 Palma, Illes Balears, Spain

² Department of Biological Chemistry and Molecular Pharmacology. Harvard Medical, School. Boston, Massachusetts 02115

³ Cancer Research-UK laboratories and Section of Cancer Cell Biology. Department of Oncology. Imperial College London, Hammersmith Hospital Campus. Du Cane Road, London W12 0NN, UK

SUMMARY

Cisplatin is a conventional chemotherapeutic agent that binds covalently to purine DNA bases and mediates cellular apoptosis. A better understanding of the downstream cellular targets of cisplatin will provide information on its mechanism of action and help to understand the mechanism of drug resistance. In this study, we have investigated the effects of cisplatin in a panel of colon carcinoma cell lines and the involvement of the PI3K/FOXO pathway in cisplatin action and resistance. Cisplatin sensitive and resistant cell lines have been characterized in cell viability, flow cytometry and clonogenic assays. The main components of the PI3K/PKB pathway, in particular FOXO3a, have been analyzed in sensitive and resistant cells upon cisplatin treatment. Interestingly, in sensitive cells cisplatin induces FOXO3a dephosphorylation and nuclear translocation, and expression of its target genes, whereas in resistant cells the effect of cisplatin on FOXO3a is incomplete. Consistent with this, PKB/FOXO signaling axis modulators Triciribine and Psammaplysene A sensitize the resistant HT29 cells to cisplatin treatment. Critically, knockdown of FOXO3a expression using siRNA rescues sensitive SW620 cells from cisplatin-induced short and long-term cell death. Together our findings suggest that FOXO3a is a relevant mediator of the cytotoxic effects of cisplatin in colon cancer cells.

Keywords

FOXO; PI3K; cisplatin; colon cancer; therapeutic targets

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer death in Western countries. Intestinal epithelial cells constitutively proliferate at a high rate to compensate for the continuous cell loss through mechanical attrition and terminal differentiation. Multiple genetic aberrations contribute to the development of biologically aggressive, clinically malignant colorectal carcinomas. The phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB, also called AKT) signaling pathway has been shown to be important for proliferation of normal and transformed intestinal epithelial cells (1,2), and has been implicated in the progression to the transformed

#To whom correspondence should be addressed.; S. Fernández de Mattos: Phone: +34-971-173004. FAX: +34-971-259501. silvia.fernandez@uib.es.

phenotype leading to colorectal carcinoma (3). Over-activation of the PI3K/PKB pathway in tumors triggers a cascade of responses, from cell growth and proliferation to survival and motility. This signaling axis plays an important role in tumorigenesis as demonstrated by the large variety of tumor-associated mutational events that affect this pathway, such as the inactivation of the tumor suppressor PTEN (4), and the frequent amplification and activation of the PI3K catalytic subunit PIK3CA (p110 α) or PKB in a large number of primary human tumors (5).

The Forkhead/winged helix box class O (FOXO) transcription factors are downstream effectors of the PI3K/PKB pathway and participate in a variety of cellular processes such as cell cycle progression, programmed cell death, stress detoxification, DNA damage repair, glucose metabolism and differentiation (reviewed in (6)). In mammals, this family of proteins consists of four members, FOXO1, FOXO3, FOXO4 and FOXO6. These factors are regulated by multiple mechanisms, including phosphorylation, acetylation and ubiquitination. Collectively, these modifications regulate the subcellular localization, transcriptional activity and stability of FOXO proteins (7–9). Among these, the phosphorylation by PKB is one of the major regulatory mechanisms by which FOXO-mediated transcription is repressed (7,10,11). PKB-phosphorylated FOXO proteins bind to 14–3–3 chaperone proteins and become sequestered in the cytoplasm, where they are unable to regulate gene expression. When active, FOXOs induce cell cycle arrest and apoptosis, negatively mediating oncogenic signaling and acting as anti-proliferative factors.

Studies in mammalian cells over recent years have identified key FOXO target genes that are involved in the regulation of cell cycle and apoptosis, such as p27^{Kip1}, cyclin D1 and cyclin D2, p130, FasL and Bim (7,12–15). Recently, we have also identified FOXO transcription factors as cellular targets of anti-tumor drugs in different cancer types, including breast cancer (16–18) and chronic myeloid leukemia (19,20). In these reports, we demonstrated that depletion of FOXO3a levels is sufficient to abrogate the apoptosis induced by the anticancer drugs, clearly confirming FOXO3a as a key regulator of cell death triggered by chemotherapeutic agents in these two types of cancer.

To date little is known about the role of FOXO transcription factors in mediating the cellular responses to drugs used in the treatment of colorectal cancer. Platinum-based compounds represent one of the main therapeutic options for colorectal cancer and their cytotoxicity is characterized by the formation of platinum adducts on DNA. However, drug resistance remains a major obstacle for effective cancer therapy, and a better understanding of its mechanism of action is urgently required. Interestingly, the PI3K/PKB/FOXO pathway has been implicated in the cellular response to cisplatin (*cis*-diamminedichloroplatinum (II), CDDP) in ovarian cancer cells (21). In this study we have analyzed the effects of the chemotherapeutic agent cisplatin in a panel of colon carcinoma cell lines. Our results show a correlation between drug sensitivity and FOXO3a activity, and suggest that FOXO3a is a relevant mediator of cisplatin-induced anti-proliferative effects in colon cancer cells.

MATERIALS AND METHODS

Cell lines and Reagents

The human colon carcinoma cell lines DLD1, HT29, SW620, SW403 and T84 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin, in a humidified incubator of 5% CO₂ at 37°C. Cisplatin (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO to a stock concentration of 10 mg/ml, and added to cultures at a final concentration of 12 μ g/ml. Triciribine phosphate (NSC 280594) was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda,

MD). Psammaplysene A was synthesized in the laboratory of Prof. J. Clardy, Harvard Medical School (Boston, MA) (22).

Proliferation assay

Cells were counted and plated at the same initial density. Following cisplatin treatment (12 µg/ml) for time periods ranging from 0 to 96 h, trypsinized cells were incubated with trypan blue. Trypan blue-negative and total cells were counted per microscope field for a total of 4 fields per condition using a Neubauer hemocytometer. The proportion of viable cells was calculated by dividing the number of viable cells by total number/field.

Clonogenic assays

For clonogenic assays, colon cancer cells were plated in 6-well plates and treated the day after with 6 or 12 µg/ml of cisplatin for a period of 6 hours. After removal of drug-containing media, cells were trypsinized and plated at low density (2000 cells/60-mm plate). Cells were then cultivated for 7–10 days and colonies stained with Crystal violet. Clones in a given area were counted for each condition. All experiments were performed three times in duplicate.

Cell cycle analysis

Cell cycle analysis was performed using propidium iodide staining as described previously (23). Briefly, cells were washed in PBS and fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained in 50 µM propidium iodide containing 5 µg/ml DNase-free RNase for 1 h, then analyzed by flow cytometry using a FACScan (Coulter Epics XL-MSL, Beckman Coulter, Fullerton, CA) and winMDI software.

Western blot analysis and antibodies

Western blot whole-cell extracts were prepared by lysing cells with lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM NaF, 1mM Na₃VO₄ and protease inhibitors “Complete” purchased from Roche) on ice for 15 min. Protein yield was quantified by Bio-Rad Dc protein assay kit (Bio-Rad). 50 µg of lysate was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Protran, Schleicher&Schuell) and specific proteins were recognized by specific antibodies. The antibodies against FOXO3a (#07–702) and phospho-FOXO3a-Thr32 (#07–695) were purchased from Millipore, phospho-JNK and JNK/SAPK antibodies were from Cell Signaling Technologies, Bim antibody was from Calbiochem (Merck) and β-catenin antibody was from Santa Cruz Biotechnology. A mouse monoclonal antibody recognizing human p27^{Kip1} was generated by Dr. Eric Lam (London, UK). The β-tubulin antibody was from PharMingen. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako), and visualized by the enhanced chemiluminiscent (ECL) detection system. Quantification of p27 and Bim protein expression was performed using the Quantity One software (BioRad).

Immunofluorescence

Cells were grown on sterile, 13-mm-diameter coverslips and fixed in 4% formaldehyde before being permeabilized in 0.01% v/v Triton X-100. Coverslips were blocked in PBS containing 3% bovine serum albumin, and antibody recognizing FOXO3a (#07–702, Millipore) was added (1:30 dilution). Specific staining was visualized with a secondary antibody conjugated to Alexa 488. Coverslips were mounted using mounting media with DAPI (Vectashield), and analyzed on a Zeiss confocal microscope with LSM meta 510 software.

Cell Viability Assay

The number of viable cells in culture was determined based on quantitation of ATP, which signals the presence of metabolically active cells. For this purpose, Cell Titer-Glo Luminiscent assay kit (Promega) was used following the manufacturer's instructions. Briefly, 5000 cells were plated on 96-well clear bottom plates, treated the next day with various concentrations of drugs and analysed 48 hours later by addition of equal volumes of media and Cell Titer-Glo Reagent. Luminiscence was detected using a multiwell scanning spectrophotometer (Plate chameleon, Hidex, Finland).

Small interfering RNA (siRNA): Transfection of psiRNA:FOXO3a into SW620 cells

The psiRNA-FOXO3a expression vector was generated by cloning small synthetic oligonucleotides encoding two complementary sequences of 19 nucleotides, TCACTGCATAGTCGATTCA, into the InvivogenpsiRNA plasmid (Autogen Bioclear, Wiltshire, UK). The cells were split 24 h prior to transfection. 10 µg of the construct was transfected into SW620 cells (6-well plate) with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The transfected cells were left to grow overnight. The day after transfection, cells were carefully washed, and 24 h later selection was started by addition of 0.5 mg/ml Zeocin to the growth medium, followed by single cell cloning and expansion for another 2 weeks. The cells were tested for the efficiency of siRNA silencing by Western blotting.

RESULTS

Cisplatin responsiveness of colon cancer cell lines

In order to characterize the mechanism of action of cisplatin in colon cancer cells, the effects of cisplatin were studied in a panel of colon carcinoma cell lines corresponding to different stages of disease progression. Cell lines DLD1, HT29, SW620, SW403 and T84 were treated with cisplatin (12 µg/ml) and the number of viable cells was analyzed for up to 96 h by the Trypan blue dye exclusion method. The results (Figure 1A) showed that these cells had differential responses to cisplatin. Treatment of SW620 and T84 cells resulted in a significant reduction in the number of growing cells, with very few viable cells left in culture 48 h after drug addition. On the contrary, HT29 and SW403 cells were more resistant to treatment, since more than 50% of cells were still viable after 96 h. The DLD1 cell line showed an intermediate sensitivity to cisplatin, with 35% of cells viable 72–96 h after treatment. In all cell lines, untreated cells grew exponentially for the time period analyzed (data not shown).

Clonogenic ability of cisplatin-treated colon cancer cell lines

In order to evaluate the long-term effects of cisplatin on the colon carcinoma cell lines, clonogenic assays were performed on HT29, SW620 and T84 cells. Briefly, cells were treated for 6 h with cisplatin, trypsinized and replated at very low density. Analysis of the stained colonies revealed that cisplatin abrogated the clonogenic ability of SW620 and T84 cells, as these cells were unable to form viable colonies following exposure to two concentrations of cisplatin (Figure 1B). Interestingly, treatment of HT29 cells with cisplatin did not significantly affect their capacity to form viable colonies. In summary, the results showed a good correlation between the outcomes of clonogenic analysis and proliferative assays regarding sensitivity to cisplatin.

Cell cycle analysis of cisplatin-treated colon cancer cell lines

To further study the effects of cisplatin on the colon cancer cell lines HT29, SW620 and T84, we analyzed the cell cycle phase distribution of cisplatin-treated cells by flowcytometry. The data shown in Figure 1C clearly indicated that there was an extensive heterogeneity in the

extent of apoptosis induced in the three cell lines analyzed. The HT29 cell line demonstrated no increase in the apoptotic fraction after cisplatin treatment as indicated by the numbers of cells with a <2N DNA content. HT29 cells did however accumulate in the S and G2/M phases, indicating a functional DNA damage checkpoint. In contrast, the drug-sensitive cell lines SW620 and T84 elicited a cell death response to cisplatin since 35–40% of cells showed a <2N DNA content following drug treatment. The data obtained using three experimental approaches (Figure 1) confirmed that the cell lines tested respond to cisplatin differently, thus allowing us to classify the SW620 and T84 cell lines as cisplatin-sensitive, and the HT29 cell line as cisplatin-resistant in the context of the panel of cell lines studied.

Expression and activity of PKB/FOXO3a in colon cancer cells

We next examined the expression of different signaling components of the PKB/FOXO3a pathway in order to determine whether differences in the expression or activity of signaling regulators could explain the heterogeneous response to cisplatin. To this end, western blot analyses were carried out in our panel of colon carcinoma cell lines. As indicated in Figure 2A, the expression levels and phosphorylation status of the survival kinase PKB show little appreciable variation between the cell lines analyzed. However, there was a significant difference in expression levels of the transcription factor FOXO3a between the three cell lines, with FOXO3a levels being particularly high in the SW403 cell line. The phosphorylation status of FOXO3a in response to PKB signaling also differs among the cell lines studied. SW403 and T84 cells showed high FOXO3a phosphorylation, while phosphorylation levels were intermediate for HT29 and SW620 cells and low in the case of DLD1 cells. The expression of β -tubulin was determined as a control for equal loading of the gels, and showed no variation.

Molecular changes induced by cisplatin treatment of SW620 and HT29 cells

To investigate the role of the PKB/FOXO3a pathway in determining the apoptotic response to cisplatin in sensitive and resistant cells, we compared the changes in protein expression following cisplatin treatment of HT29 and SW620 cells for various times (Figure 2B). In the cisplatin-sensitive SW620 cell line treatment with cisplatin induced a rapid and sustained change in the phosphorylation status of PKB and its substrate FOXO3a. The complete dephosphorylation of PKB and the very pronounced dephosphorylation of FOXO3a for up to 48 h after treatment indicate that cisplatin inhibits the survival kinase PKB and induces the activity of the FOXO3a transcription factor in SW620 cells. On the contrary, in the resistant cell line HT29 the effect of cisplatin on FOXO3a phosphorylation was less pronounced. Although PKB phosphorylation was reduced by cisplatin, it was not totally abrogated and significantly recovered by 48 h. Similarly, FOXO3a phosphorylation was partially affected by cisplatin but could not be completely blocked during the time period analyzed. Interestingly, the cell cycle inhibitor p27^{Kip1}, a direct target of FOXO3a, was also differentially affected by cisplatin treatment, being up-regulated in response to cisplatin in SW620 cells and showing little changes in HT29 cells (Fig. 2B and 2C). Similarly, the pro-apoptotic protein Bim, which has been reported to be a direct target of FOXO3a (20), was induced in SW620 cells 16 h after drug addition, while its levels remained almost undetectable in HT29 cells (Fig. 2B and 2C). These results correlate with the pattern of FOXO3a dephosphorylation and activation, and suggest that in HT29 cells cisplatin is unable to activate FOXO3a sufficiently to transactivate the expression of its main targets such as p27^{Kip1} and Bim.

We then studied if other signaling pathways were also differentially affected by cisplatin in these two cell lines. The JNK pathway has been shown to be important for cisplatin-induced apoptosis in various cellular models (24,25), and JNK has also been demonstrated to activate FOXO proteins in response to stress (26–28). We thus analyzed the phosphorylation status and expression levels of JNK in both cell lines, and found that cisplatin induced a sustained phosphorylation of JNK in both the HT29 and the SW620 cells without affecting its expression

levels. β -catenin is a multifunctional protein that has been extensively implicated in the development of colon cancer, and has been demonstrated to regulate FOXO3a function under conditions of oxidative stress (29). We thus analyzed the expression of β -catenin during cisplatin treatment of HT29 and SW620 cell lines, and the results showed that its levels were slightly up-regulated by cisplatin in both cases. In the SW620 cell line, β -catenin expression was lost 48 h after cisplatin addition, probably due to the high levels of cell death accomplished at this late time point. In summary, considering the major differences relate to the ability of cisplatin to alter the activity of the PKB/FOXO3a pathway, these results suggest a plausible role for FOXO3a in determining the cellular response to cisplatin.

FOXO3a subcellular localization is affected by cisplatin treatment in T84 cells

FOXO proteins are transcription factors and their activity is thus highly influenced by their subcellular localization, a process which is tightly regulated. We next investigated if cisplatin treatment relocates the FOXO3a protein to the nucleus. To this end, the cisplatin-sensitive T84 cells were treated with cisplatin for different periods of time, fixed and the intracellular localization of FOXO3a was analyzed by immunocytochemical staining. The results clearly showed that in untreated cells FOXO3a resided in the cytoplasm, with nuclear staining negligible (Figure 3). Interestingly, cisplatin treatment induced a gradual change in the subcellular localization of FOXO3a, with a proportion of the protein being translocated to the nucleus in response to drug addition (8 and 24 h). This correlates with activation of FOXO3a in response to cisplatin, and coincides with the de-phosphorylation that occurs in SW620 cells. These immunocytochemistry results support our hypothesis that nuclear translocation of the FOXO3a transcription factor may contribute to FOXO3a activation and induction of cell death in cisplatin-sensitive cells.

Chemical modulators of PKB and FOXO activity induce cell death and sensitize cells to cisplatin

Since the differential effect of cisplatin on the PI3K pathway could be due to an altered function of this pathway in HT29 cells, we studied the response of sensitive and resistant cells to chemical modulators of the PKB/FOXO signaling axis. For this purpose, we used the PKB small molecule inhibitor Triciribine-phosphate (30) and the specific FOXO nuclear export inhibitor Psammaplysene A (22), that has been shown to compensate for PTEN loss in PTEN-deficient cells (31). Different concentrations of these compounds were used in cell viability assays of HT29 and SW620 cells. The results shown in Figure 4A demonstrate that treatment with Triciribine-phosphate reduced cell viability to less than 50% compared with control cells, and that increasing concentrations of Psammaplysene A had a profound effect on cell viability of both cell lines. These results suggest that the PKB/FOXO signaling axis is functional in both the cisplatin-sensitive and -resistant cell lines, since two different chemical modulators are able to significantly compromise cell viability in HT29 and SW620 cells.

We were also able to detect the differential response to cisplatin of HT29 and SW620 cells in cell viability assays (Figure 4A). As both cell lines were similarly affected by the inhibitors regardless of their responsiveness to cisplatin, we next wanted to assess whether simultaneous treatment with cisplatin and Triciribine-phosphate or Psammaplysene A could have an additive effect. We compared cell viability of HT29 and SW620 cells upon cisplatin treatment with the viability of cells treated with cisplatin together with Triciribine-phosphate (10 μ M) or Psammaplysene A (0.5 μ M). In both cell lines, suboptimal concentrations of Triciribine or Psammaplysene A showed a cooperative effect with the different concentrations of cisplatin tested (Figure 4B). Although the viability of both cell lines after treatment with cisplatin alone was remarkably different, cotreatment with Triciribine or Psammaplysene A not only had a significant effect on both cell lines, but also rendered resistant HT29 cells to behave similarly to SW620 cells in terms of cell viability. These results suggest that concomitant treatment with

chemical modulators of the PKB/FOXO pathway can overcome cisplatin resistance. Also, the effect of cisplatin on sensitive cells can be further enhanced by modulation of PKB/FOXO signaling.

Silencing of FOXO3a in SW620 cells impairs the cellular response to cisplatin

In order to confirm the relevance of the FOXO3a factor in the cellular response to cisplatin, we next decided to perform gene silencing experiments. To this end, derivatives of the cisplatin-sensitive cell line SW620 were generated in which expression of the FOXO3a protein was abrogated by the small interfering RNA (siRNA) technology. SW620 cells were transduced with the psiRNA-FOXO3a construct, and zeocin-resistant cells were expanded and analyzed for FOXO3a expression. As shown in Figure 5A, the resistant cells showed a complete silencing of FOXO3a expression.

We next performed cell growth assays of SW620 cells together with the newly generated FOXO3a-deficient SW620 cells (psiRNA-FOXO3a). Cells were treated with cisplatin for 24 and 48 h, and the number of viable cells was analyzed by the Trypan blue dye exclusion method. As shown in Figure 5B, cisplatin affected both cell lines differently. In the parental cell line, only 34% of cells were viable 48 h after drug addition, whereas viability of cells lacking FOXO3a expression was more than two fold higher (72%). This suggested that the absence of FOXO3a expression impaired the efficacy of cisplatin treatment in SW620 cells.

In order to confirm this hypothesis, clonogenic assays were performed in both cell lines, together with the cisplatin-resistant cell line HT29 as a control. As shown in Figure 5C, HT29 and SW620 cells retained the previously observed difference (see Fig. 1B) regarding the long-term effects of cisplatin. Only the parental SW620 cells were affected in their ability to form viable clones following cisplatin treatment, while SW620 cells lacking FOXO3a expression behaved like cisplatin-resistant cells in clonogenic assays, with no differences in the number of viable clones between the untreated cells and cells exposed to cisplatin.

Finally, this difference in the response to cisplatin depending on the presence of FOXO3a was further confirmed by flow cytometry analysis of SW620 cells and the psiRNA-FOXO3a derivatives (Figure 5D). The data obtained revealed that, while cisplatin treatment of SW620 cells for 48 h induced cell death in 37% of the cell population, the level of cell death in cells lacking FOXO3a expression was only 10%. This sharp contrast in viability between parental and FOXO3a-depleted SW620 cells highlights the biological relevance of FOXO3a as a mediator of cisplatin signaling and sensitivity.

DISCUSSION

The PI3K pathway plays an important role in the development and progression of colon cancer. The catalytic subunit of the class IA PI3K, PI3KCA, has been reported to be frequently mutated in colorectal tumors (32). Interestingly, human colon carcinoma cells engineered to express constitutively active PI3KCA show a specific effect on the regulation of FOXO3a and FOXO1, but not on other PKB substrates (33). Recently, the extent of PKB activation has been correlated with the progression of colorectal neoplasia (34). Despite the importance of PI3K/PKB/FOXO signaling in colon cancer, little has been established regarding the role of components of this pathway in the response of colorectal cancer cells to therapeutic strategies. Platinum compounds are routinely used in treatment of colorectal cancer, but acquired resistance diminishes the efficacy of these drugs. Accumulated evidence supports a role for altered signaling in mediating resistance. The main goal of this study was to evaluate the contribution of the PI3K pathway and the transcription factor FOXO3a to the effects of cisplatin treatment in colon cancer cells.

We have screened a panel of colon carcinoma cell lines and identified two cell lines, SW620 and T84 to be sensitive to cisplatin treatment. Using these two cell lines as well as the resistant cell line HT29, we have shown that cisplatin differentially affects FOXO3a dephosphorylation and activation, which ultimately dictate the overall cellular response to cisplatin. In sensitive cells, cisplatin causes apoptosis and affects long-term cell survival, which is associated with FOXO3a dephosphorylation and nuclear translocation, whereas in the resistant cells FOXO3a dephosphorylation is incomplete and is thus unable to transactivate expression of targets such as p27^{Kip1} and Bim to cause cell cycle arrest and cell death. The failure of cisplatin to significantly reduce the phosphorylation of FOXO3a in HT29 cells, despite an inhibitory effect on PKB, could be attributed to the activity of other kinases which can also phosphorylate FOXO3a on the same residue (Thr32) such as serum- and glucocorticoid-inducible kinase (SGK). Interestingly, inhibition of PKB with Triciribine or activation of FOXO with the specific nuclear-export inhibitor Psammaplysene A have a dramatic effect on cellular viability in both cell lines, which suggests that the PKB/FOXO axis is functional, and that the ability of cisplatin to activate FOXO signaling is impaired in cisplatin-resistant cells. Accordingly, cell viability analyses show that a suboptimal dose of Triciribine or Psammaplysene A enhance sensitivity to cisplatin. Moreover, these chemical inhibitors resensitize resistant colon carcinoma cells to cisplatin. Taken together, these data clearly demonstrate the capacity of chemical modulators of the PKB/FOXO pathway to cooperate with cisplatin in the inhibition of colon cancer cell growth. Furthermore, silencing of endogenous FOXO3a expression by siRNA partially rescued the sensitive cell line SW620 from undergoing apoptosis in response to cisplatin. Interestingly, FOXO3a-depleted cells underwent cell cycle arrest after cisplatin treatment, similarly to previous results in HT29 resistant cells, indicating that FOXO3a is predominantly involved in the induction of apoptosis in response to cisplatin treatment.

However, as silencing of FOXO3a expression does not completely abrogate the apoptotic effect of cisplatin, it is reasonable to conclude that other mechanisms must be involved in determining the response of colon cancer cells to cisplatin treatment. Notably, other proteins directly involved in PI3K signaling have been implicated in cisplatin therapy in other types of cancers. In lung cancer cells, cisplatin resistance has been associated with AKT1 (PKB α) over-expression and gene amplification. AKT1 forced expression is sufficient to render lung cancer cells cisplatin resistant, and AKT1 inhibition reverses the cisplatin-resistant cells to be cisplatin sensitive (35). Furthermore, the authors prove that AKT activation is highly related to cisplatin chemosensitivity in human tumor tissues. Very recently, the microRNA miR-214 has been shown to induce cisplatin resistance of ovarian cancer cells negatively regulating PTEN translation and hence activating the AKT pathway (36). These reports support the idea that combination therapy with cisplatin and PI3K inhibitors would increase the therapeutic effects of cisplatin.

Mounting evidence has implicated FOXO proteins as therapeutic targets. We and others have reported a role for FOXO3a as an indirect target of the chemotherapeutic drugs paclitaxel and KP372-1 (a multiple kinase inhibitor) in breast carcinoma cell lines (16,17) and acute myeloid leukemia cells (37), respectively. Both drugs activate FOXO3a by reducing PKB activity. Paclitaxel is also able to activate JNK, and this also modulates the activity and stability of FOXO3a (17). We have also shown that FOXO3a is important for imatinib-induced apoptosis of chronic myeloid leukemia cell lines that express the BCR-ABL oncoprotein (19,20). In these cells imatinib induces FOXO3a activity leading to cyclin D2 down-regulation, cell cycle arrest and Bim-dependent apoptosis (19,20). Inhibition of the epidermal growth factor receptors (EGFR) represents a valuable therapeutic strategy against breast, prostate, lung, and ovarian cancer amongst others. Interestingly, in breast cancer cells blockade of EGFR by antibodies (such as trastuzumab or cetuximab) induces FOXO3a activity and expression of the pro-apoptotic *BNIP3L* gene (38). Also, it has been demonstrated that small molecule inhibitors of

EGFR such as gefitinib (currently in clinical trials) induce proliferative arrest of breast cancer cells through dephosphorylation and activation of FOXO3a (18).

We have shown that treatment of colon cancer cells with the combination of cisplatin plus Triciribine or Psammaplysene A caused a more marked reduction of cell viability than treatment with either agent alone. Similar results have been reported in the pancreatic cancer cell line Panc-1 and the breast cancer cell line MDA-MB-468, in which inhibition of PI3K by LY294002 sensitized cells to cisplatin-induced cell death (39). It has also been reported that inhibition of PI3K with wortmannin increases the efficacy of cisplatin in *in vivo* ovarian cancer models (40). Our results further confirm that simultaneous inhibition of the PKB/FOXO cascade enhances the ability of cisplatin to induce cell death, and provide valuable information for the development of treatment protocols for colon cancer through targeting the PI3K/PKB/FOXO cell survival pathway. Interestingly, the novel antitumoral drug 17-AAG (inhibitor of the molecular chaperone Hsp90) has been reported to synergistically interact with cisplatin in colon cancer cell lines (41), highlighting the need for targeted therapies to improve the current standard of colon cancer treatment.

Collectively, our data suggest that FOXO3a performs an important function in the cytotoxic effect of cisplatin, thereby implying that FOXO3a may be one of the cellular factors determining the cellular sensitivity to the drug. These data also suggest that inhibition of PKB or FOXO3a nuclear export can resensitize resistant colon carcinoma cells to cisplatin. On the basis of these observations, we propose that one way to enhance the cytotoxicity of cisplatin in sensitive cells, and to overcome drug resistance in unresponsive cells, is to target the PI3K/PKB/FOXO signaling pathway with specific inhibitors in combination with cisplatin.

Acknowledgments

GRANT SUPPORT: This work was sponsored by the “Ramón y Cajal” Program of the Ministerio de Educación y Ciencia (Spain) and by Junta de Balears-AECC. S. F. de M. and P. V. are fellows of the “Ramón y Cajal” Program (Ministerio de Educación y Ciencia, Spain). E.W-F. L. is supported by Cancer Research-UK.

We are grateful to Dr. Catalina Crespi from the Research Unit in Hospital de Son Dureta for help with flow cytometry analysis.

ABBREVIATIONS

PI3K	phosphoinositide-3-kinase
FOXO	Forkhead/winged helix box class O
EGFR	epidermal growth factor receptor
SGK	serum- and glucocorticoid-inducible kinase
siRNA	small interfering RNA

References

1. Sheng H, Shao J, Townsend CM Jr, Evers BM. Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells. *Gut* 2003;52:1472–8. [PubMed: 12970141]

2. Shao J, Evers BM, Sheng H. Roles of phosphatidylinositol 3'-kinase and mammalian target of rapamycin/p70 ribosomal protein S6 kinase in K-Ras-mediated transformation of intestinal epithelial cells. *Cancer Res* 2004;64:229–35. [PubMed: 14729629]
3. Khaleghpour K, Li Y, Banville D, Yu Z, Shen SH. Involvement of the PI 3-kinase signaling pathway in progression of colon adenocarcinoma. *Carcinogenesis* 2004;25:241–8. [PubMed: 14578160]
4. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000;100:387–90. [PubMed: 10693755]
5. Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci U S A* 2001;98:10983–5. [PubMed: 11572954]
6. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer* 2007;7:847–59. [PubMed: 17943136]
7. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999;96:857–68. [PubMed: 10102273]
8. Motta MC, Divecha N, Lemieux M, et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell* 2004;116:551–63. [PubMed: 14980222]
9. van der Horst A, de Vries-Smits AM, Brenkman AB, et al. FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat Cell Biol* 2006;8:1064–73. [PubMed: 16964248]
10. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* 1999;398:630–4. [PubMed: 10217147]
11. Tang ED, Nunez G, Barr FG, Guan KL. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem* 1999;274:16741–6. [PubMed: 10358014]
12. Medema RH, Kops GJPL, Bos JL, Burgering BMT. AFX-like Forkhead transcription factors mediate cell cycle regulation by Ras and PKB via p27. *Nature* 2000;404:782–7. [PubMed: 10783894]
13. Schmidt M, Fernandez de Mattos S, van der Horst A, et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol* 2002;22:7842–52. [PubMed: 12391153]
14. Kops GJ, Medema RH, Glassford J, et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 2002;22:2025–36. [PubMed: 11884591]
15. Dijkers PF, Medemadagger RH, Lammers JW, Koenderman L, Coffey PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* 2000;10:1201–4. [PubMed: 11050388]
16. Sunter A, Fernandez de Mattos S, Stahl M, et al. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 2003;278:49795–805. [PubMed: 14527951]
17. Sunter A, Madureira PA, Pomeranz KM, et al. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. *Cancer Res* 2006;66:212–20. [PubMed: 16397234]
18. Krol J, Francis RE, Albergaria A, et al. The transcription factor FOXO3a is a crucial cellular target of gefitinib (Iressa) in breast cancer cells. *Mol Cancer Ther* 2007;6:3169–79. [PubMed: 18089711]
19. Fernandez de Mattos S, Essafi A, Soeiro I, et al. FoxO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism. *Mol Cell Biol* 2004;24:10058–71. [PubMed: 15509806]
20. Essafi A, Fernandez de Mattos S, Hassen YA, et al. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* 2005;24:2317–29. [PubMed: 15688014]
21. Arimoto-Ishida E, Ohmichi M, Mabuchi S, et al. Inhibition of Phosphorylation of a Forkhead Transcription Factor Sensitizes Human Ovarian Cancer Cells to Cisplatin. *Endocrinology* 2003;30:30.
22. Schroeder FC, Kau TR, Silver PA, Clardy J. The psammmaplysenes, specific inhibitors of FOXO1a nuclear export. *J Nat Prod* 2005;68:574–6. [PubMed: 15844952]
23. Williams CD, Linch DC, Watts MJ, Thomas NS. Characterization of cell cycle status and E2F complexes in mobilized CD34+ cells before and after cytokine stimulation. *Blood* 1997;90:194–203. [PubMed: 9207453]

24. Sanchez-Perez I, Murguía JR, Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene* 1998;16:533–40. [PubMed: 9484843]
25. Mansouri A, Ridgway LD, Korapati AL, et al. Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells. *J Biol Chem* 2003;278:19245–56. [PubMed: 12637505]
26. Wang MC, Bohmann D, Jasper H. JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* 2005;121:115–25. [PubMed: 15820683]
27. Oh SW, Mukhopadhyay A, Svrikapa N, Jiang F, Davis RJ, Tissenbaum HA. JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* 2005;102:4494–9. [PubMed: 15767565]
28. Essers MA, Weijzen S, de Vries-Smits AM, et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J* 2004;23:4802–12. [PubMed: 15538382]
29. Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. Functional interaction between beta-catenin and FOXO in oxidative stress signaling. *Science* 2005;308:1181–4. [PubMed: 15905404]
30. Yang L, Dan HC, Sun M, et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res* 2004;64:4394–9. [PubMed: 15231645]
31. Kau TR, Schroeder F, Ramaswamy S, et al. A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. *Cancer Cell* 2003;4:463–76. [PubMed: 14706338]
32. Parsons DW, Wang TL, Samuels Y, et al. Colorectal cancer: mutations in a signalling pathway. *Nature* 2005;436:792. [PubMed: 16094359]
33. Samuels Y, Diaz LA Jr, Schmidt-Kittler O, et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* 2005;7:561–73. [PubMed: 15950905]
34. Saglam O, Garrett CR, Boulware D, et al. Activation of the serine/threonine protein kinase AKT during the progression of colorectal neoplasia. *Clin Colorectal Cancer* 2007;6:652–6. [PubMed: 17945038]
35. Liu LZ, Zhou XD, Qian G, Shi X, Fang J, Jiang BH. AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. *Cancer Res* 2007;67:6325–32. [PubMed: 17616691]
36. Yang H, Kong W, He L, et al. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* 2008;68:425–33. [PubMed: 18199536]
37. Zeng Z, Samudio IJ, Zhang W, et al. Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia. *Cancer Res* 2006;66:3737–46. [PubMed: 16585200]
38. Real PJ, Benito A, Cuevas J, et al. Blockade of epidermal growth factor receptors chemosensitizes breast cancer cells through up-regulation of Bnip3L. *Cancer Res* 2005;65:8151–7. [PubMed: 16166289]
39. Winograd-Katz SE, Levitzki A. Cisplatin induces PKB/Akt activation and p38(MAPK) phosphorylation of the EGF receptor. *Oncogene* 2006;25:7381–90. [PubMed: 16785992]
40. Ohta T, Ohmichi M, Hayasaka T, et al. Inhibition of phosphatidylinositol 3-kinase increases efficacy of cisplatin in in vivo ovarian cancer models. *Endocrinology* 2006;147:1761–9. [PubMed: 16396982]
41. Vasilevskaya IA, Rakitina TV, O'Dwyer PJ. Quantitative effects on c-Jun N-terminal protein kinase signaling determine synergistic interaction of cisplatin and 17-allylamino-17-demethoxygeldanamycin in colon cancer cell lines. *Mol Pharmacol* 2004;65:235–43. [PubMed: 14722256]

Figure 1. Cisplatin responsiveness of colon cancer cell lines

A, Growth curves of colon cancer cell lines after cisplatin treatment. The indicated colon cancer cell lines were treated with 12 $\mu\text{g/ml}$ of cisplatin for various time periods, as indicated, and the viable cells were quantified by the Trypan blue dye exclusion method. Shown is the number of viable cells per each condition. **B, Clonogenic analysis of colon cancer cell lines after cisplatin treatment.** The indicated colon cancer cell lines were treated with 6 or 12 $\mu\text{g/ml}$ cisplatin for 6 hours, trypsinized and plated in duplicates at low density (2000 cells/60-mm plate). After two weeks formed colonies were stained with Crystal violet. Clones in a given area were counted for each condition. Represented is the percentage of viable clones after cisplatin treatment respective to untreated cells. Data is means $\pm$ S.E.M. from three independent determinations. **C, Cell cycle analysis of colon cancer cells treated with cisplatin.** The indicated colon cancer cell lines were treated with 12 $\mu\text{g/ml}$ cisplatin for 24 hours, fixed in ethanol, stained with propidium iodide, and DNA content determined by flow cytometry. Top left panel shows gating for each cell cycle phase (M1: $<2N$, M2: G0/G1, M3: S, M4: G2/M). Same gating has been applied to all histograms. The percentage of cells in each phase of the cell cycle is also indicated.

Figure 2. Analysis of the PKB/FOXO3a pathway in response to Cisplatin

A, Expression of PKB/FOXO3a in colon cancer cells. Whole cell lysates were prepared from the panel of colon cancer cell lines. The expression of both phosphorylated and total FOXO3a, and phosphorylated and total PKB was analyzed by Western blotting. Also shown is a blot for β -tubulin as a loading control. *B*, Analysis of molecular changes induced by Cisplatin in SW620 and HT29 cells. Whole cell lysates were prepared from HT29 and SW620 cells treated with 12 μ g/ml Cisplatin for the times indicated. The expression and phosphorylation of the indicated proteins were analyzed by Western blotting. Also shown is a blot for β -tubulin as a loading control. MW, molecular weight. *C*, Quantitative analysis of p27 and Bim protein expression.

Graphs show quantification of p27 and Bim protein expression after normalizing the data to $t = 0$ h. Values have been corrected for tubulin expression for each independent time point.

Cisplatin (h)

Figure 3. Subcellular localization of FOXO3a in T84 cells

T84 cells were cultured on sterile coverslips and treated for 8 or 24 hours with 12 $\mu\text{g/ml}$ Cisplatin, before being fixed in 4% formaldehyde. FOXO3a was visualized with a rabbit polyclonal antibody followed by the addition of ALEX488 (green) labeled anti-rabbit antisera. Mounting media with DAPI (blue) was applied to visualize the cytoplasm and nuclei, respectively.

Figure 4. Cell viability assay of SW620 and HT29 cells after treatment with cisplatin, Triciribine and Psammaplysen A

A and B, Cell viability of SW620 and HT29 cells was measured using the Cell Titer-Glo luminescent cell viability assay kit. Cells were treated with Triciribine-phosphate (TCB), Psammaplysen A (PsA) and/or cisplatin at the indicated concentrations (in *B*, Triciribine 10 μ M, Psammaplysen A 0.5 μ M) and incubated for 48 h, followed by addition of Cell Titer-Glo Reagent and detection of luminescence with a multiwell scanning spectrophotometer. Cell viability is represented as a percentage relative to untreated cells, and data is means $\pm$ S.E.M. from three independent determinations performed in duplicate.

Figure 5. Knock-down of FOXO3a expression in SW620 cells alters cisplatin response^A, *FOXO3a silencing in SW620 cells*

SW620 cells were transfected with the psiRNA-FOXO3a plasmid containing oligonucleotides that encode two complementary sequences against human FOXO3a. Transfected cells were selected by addition of zeocin to the growing media. The selected cells were expanded, and depletion of FOXO3a expression was evaluated by western blot. **B**, *Growth curves of SW620 and psiRNA-FOXO3a cells after cisplatin treatment*. The indicated cell lines were treated with 12 µg/ml of cisplatin for various time periods, as indicated, and the viable cells were quantified by the Trypan blue dye exclusion method. Shown is the percentage of viable cells per each condition relative to untreated cells. Data is means ± S.E.M. from three independent determinations. **C**, *Clonogenic analysis after cisplatin treatment*. The indicated cell lines were treated with 12 µg/ml cisplatin for 6 hours, trypsinized and plated in duplicates at low density (2000 cells/60-mm plate). After two weeks formed colonies were stained with Crystal violet. Clones in a given area were manually counted for each condition. Represented is the percentage of viable clones after cisplatin treatment relative to untreated cells. Data is means ± S.E.M. from three independent determinations. **D**, *Cell cycle analysis of cells treated with cisplatin*. The indicated cell lines were treated with 12 µg/ml cisplatin for 48 hours, fixed in ethanol, stained with propidium iodide, and DNA content determined by flow cytometry. Top left panel shows gating for each cell cycle phase (M1: <2N, M2: G0/G1, M3: S, M4: G2/M). Same gating has been applied to all histograms. The percentage of cells in each phase of the cell cycle is also indicated.