Altered Expression of c-IAP1, Survivin, and Smac Contributes to Chemotherapy Resistance in Thyroid Cancer Cells

Elena Tirrò,[1] Maria Letizia Consoli,[1] Michele Massimino,[1] Livia Manzella,[1] Francesco Frasca,[2] Laura Sciacca,[2] Luisa Vicari,[3] Giorgio Stassi,[4] Luigi Messina,[1] Angelo Messina,[1] and Paolo Vigneri[1]

[1]Department of Biomedical Sciences, Section of General Pathology and [2]Department of Internal and Specialistic Medicine, Section of Endocrinology, University of Catania; [3]Department of Experimental Oncology, Istituto Oncologico del Mediterraneo, Catania; and [4]Department of Surgical and Oncological Sciences, University of Palermo, Palermo, Italy

Abstract

Resistance to chemotherapy predicts an unfavorable outcome for patients with radioiodine-insensitive thyroid cancer. To investigate the mechanisms underlying this resistance, we evaluated the expression of four different inhibitor of apoptosis proteins, and their antagonist, Smac, in thyroid cancer cells that survived 48 hours of exposure to cisplatin, doxorubicin, or taxol. We found high levels of c-IAP1 after cisplatin treatment and increased expression of survivin following exposure to doxorubicin. Cells that endured treatment with taxol showed reduced expression of Smac and released minimal amounts of this protein from the mitochondria. Down-regulation of c-IAP1 and survivin increased the cytotoxicity of cisplatin and doxorubicin, whereas overexpression of Smac improved the efficacy of taxol. Finally, thyroid cancer cells permanently resistant to doxorubicin or cisplatin showed increased expression of c-IAP1 and survivin, respectively. However, silencing of these proteins by RNA interference restored sensitivity to doxorubicin and cisplatin. Thus, in thyroid cancer cells, early resistance to chemotherapeutic agents requires high levels of c-IAP1 and survivin and low levels of Smac. Furthermore, increased expression of c-IAP1 and survivin contributes to the acquisition of permanent resistance to cytotoxic compounds. (Cancer Res 2006; 66(8): 4263-72)

Introduction

The inhibitor of apoptosis (IAP) family comprises a group of eight proteins (Apollon, c-IAP1, c-IAP2, ILP-2, Livin, NAIP, survivin, XIAP) that play a pivotal role in the negative regulation of cell death (1). All IAPs present a common structural feature: they display one or more Baculovirus IAP repeat domains that are directly responsible for the antiapoptotic function of these proteins (2).

Although the exact mechanisms responsible for IAP-mediated suppression of cell death have yet to be fully elucidated, convincing evidence suggests that c-IAP1, c-IAP2, and XIAP inhibit cell death by binding to caspase-3, caspase-7, and caspase-9, and abrogating their proapoptotic function (1). Further evidence has suggested that IAP proteins presenting a zinc-binding RING domain can function as E3 ubiquitin ligases, targeting proapoptotic molecules to proteasome-mediated degradation (3). Examples include XIAP-induced proteasomal degradation of caspase-3 and Apollon-mediated ubiquitylation and degradation of caspase-9 (4, 5). Interestingly, it seems that expression of a RING domain is also responsible for IAP autoubiquitylation and degradation, therefore suggesting that an intricate interplay of different pathways will determine if IAP proteins induce ubiquitylation of target substrates (blocking cell death) or autoubiquitylation (favoring cell death; ref. 3).

The second mitochondrial activator of caspases (Smac) protein is an antagonist of several IAPs, including c-IAP1, c-IAP2, survivin, and XIAP (6). In healthy cells, Smac localizes to the intermembrane space of the mitochondria. However, upon induction of cell damage, the protein is promptly released to the cytosol where it binds to different IAPs via an IAP-binding motif located on its NH_2 terminus. The ensuing disruption of the physical interaction between IAPs and caspases enables the latter proteins to exert their protease activity and induce cell death (7). Recent evidence suggesting that XIAP (and possibly other RING-containing IAPs) may ubiquitylate Smac, targeting it to the proteasome, suggest a further level of regulation in the delicate stoichiometric balance between IAP proteins and Smac (8).

Most epithelial thyroid carcinomas derive from the malignant transformation of follicular cells (9, 10). Usually, these neoplasias present a favorable prognosis if properly diagnosed and treated with a combined approach that associates radical surgery with radioiodine ablation of possible residual cancerous lesions, and pharmacologic suppression of thyroid-stimulating hormone, a specific growth factor for thyroid cells (11).

Unfortunately, a small number of patients will develop a more aggressive form of the disease characterized by poorly differentiated thyroid cancer (PDTC) cells that have lost their capacity to uptake and retain radioactive iodine and are therefore insensitive to radioiodine treatment (12). Likewise, individuals affected by undifferentiated (anaplastic) thyroid carcinoma (UTC) are always unresponsive to radioiodine because their neoplastic cells have lost expression and/or function of the sodium-iodine symporter, responsible for iodine uptake (13). Although PDTCs and UTCs represent <10% of all thyroid carcinomas, they are responsible for the majority of thyroid cancer–related deaths (12, 13).

A combination of chemotherapeutic drugs is presently the best therapeutic approach for patients affected by radioiodine-insensitive thyroid carcinomas (14). However, a large number of these individuals present refractoriness to chemotherapy and are therefore confronted with a dismal prognosis. Whereas many different mechanisms have been directly or indirectly invoked to

Note: E. Tirrò and M.L. Consoli contributed equally to this work.

Requests for reprints: Paolo Vigneri, Department of Biomedical Sciences, Section of General Pathology, University of Catania, Via Androne 83, 95124 Catania, Italy. Phone: 39-95-312389; Fax: 39-95-715-1928; E-mail: pvigneri@libero.it.

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explain chemotherapy resistance by neoplastic cells (15), recent evidence suggests that reduced rates of cell death following exposure to antiblastic drugs represent a critical step in the development of chemoresistance (16).

We have examined the role of four members of the IAP family (c-IAP1, c-IAP2, survivin, and XIAP) and of their antagonist Smac in the resistance to chemotherapy of PDTC or UTC cells. We found increased expression of c-IAP1 and survivin and reduced levels of Smac in thyroid cancer cells that survived a 48-hour exposure to *cis*-diammine-dichloro-platinum (CDDP), doxorubicin or taxol. In these cells, we also observed a minimal cytoplasmic relocalization of Smac. Silencing of c-IAP1 and survivin or overexpression of Smac increased thyroid cancer cell sensitivity to chemotherapy. In addition, thyroid cancer cells permanently resistant to doxorubicin showed increased expression of c-IAP1, whereas cells permanently resistant to CDDP presented high levels of survivin. Noticeably, silencing of c-IAP1 and survivin restored cell sensitivity to doxorubicin or CDDP.

These findings suggest that the initial lack of death observed in PDTC or UTC cells after treatment with chemotherapeutic agents requires high levels of c-IAP1 and survivin and a reduced expression of Smac. Furthermore, c-IAP1 and survivin are also directly involved in the permanent resistance to doxorubicin or CDDP of thyroid cancer cells.

Materials and Methods

Cell lines and primary cultures. Human thyroid cancer cells ONCO-DG1, BC-PAP (derived from papillary carcinomas), WRO, FTC-133 (derived from follicular carcinomas), and KAT-4 (derived from anaplastic carcinomas) were grown in RPMI 1640 (Sigma, St. Louis, MO), whereas SW-1736 and 8305C (immortalized from anaplastic carcinomas) were cultured in MEM (Sigma). Tissue culture media were supplemented with 10% fetal bovine serum (Cambrex, East Rutherford, NJ), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L of glutamine (all from Sigma).

Primary human thyrocytes (normal or neoplastic) were obtained from surgical specimens that were initially fragmented with a scalpel and subsequently digested for 1 hour in DMEM low glucose (Sigma) containing 1 mg/mL type V collagenase (Sigma) in a 37°C water bath with agitation. The cell suspension was then centrifuged and the pellet resuspended in RPMI 1640 with decreasing doses (from 10% to 1%) of stripped fetal bovine serum, penicillin, streptomycin, glutamine (at the concentrations indicated above) and 2.5 μg/mL amphoterycin B (Sigma). All primary cultures were analyzed by Immunofluorescence using an antibody against cytokeratin (Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom) that stains exclusively epithelial cells. The purity of each preparation was >95%.

Drug treatments and resistant clones. CDDP, doxorubicin and taxol were purchased from Sigma and resuspended in sterile water (CDDP and taxol) or PBS (doxorubicin).

WRO cells resistant to CDDP (5 μg/mL), doxorubicin (1 μmol/L) or taxol (5 μmol/L) were established by continuous exposure to increasing doses of the drugs over a period of 30 to 40 weeks.

Survival assays. Cell lines were plated in triplicates, exposed to the indicated concentrations of chemotherapeutic drugs for 24, 48 or 72 hours and apoptotic cells were then scored by trypan blue staining.

Alternatively, cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Calbiochem, San Diego, CA). Briefly, 1.5×10^3 cells were plated in a 96 multiwell dish and treated with chemotherapeutic drugs for 24, 48 or 72 hours. 20 μL of 5 mg/mL MTT reagent in PBS were added to each well at the appropriate time point. After 4 hours of incubation at 37°C, the diluted MTT reagent was removed, and 100 μL of DMSO + 2.5% improved minimal essential medium were added to each well. Absorbance was measured at 545 nm.

In some experiments, apoptotic cells were evaluated by immunofluorescence, scoring the number of cells exhibiting fragmented nuclei after their DNA was stained with the Hoechst 33258 dye (Sigma).

RNA extraction and RT-PCR. Total RNA was extracted using the Trizol reagent (Invitrogen, Paisley, United Kingdom) and 2 μg of RNA were then reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). One microliter of the synthesized cDNA was then used for PCR amplifications employing a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) and Taq DNA polymerase from Invitrogen. Primers used for the different reactions are listed below:

c-IAP1 forward CCCAGGTCCCTCGTATCAAA
c-IAP1 reverse AAACCAGCTCTTGCCAATTC
c-IAP2 forward TGAAAAGCGCCAACAC
c-IAP2 reverse ACTAGAGGGCCAGTTAAAGA
Survivin forward CTCAAGGACCACCGCATCTC
Survivin reverse GGCTGCCATGGATTGAGG
XIAP forward TTTTAACAGTTTTGAAGGAT
XIAP reverse GCCCAAAACAAAGAAGCAAT
Smac forward GTGTGTTCCTGTTGTGGCT
Smac reverse GCCCAAAACAAAGAAGCAAT
NCOA4 forward ATTGAAGAAATTGCAGGCTC
NCOA4 reverse TGGAGAAGAGGAGCTGTATCT

Immunoblotting and subcellular fractionation. Cell pellets were resuspended in isotonic buffer [25 mmol/L Trizma base (pH 8.5), 10 mmol/L NaCl, 7 mmol/L β-mercaptoethanol, 1× protease inhibitor cocktail (Roche, Indianapolis, IN)], sonicated and harvested by centrifugation at 14,000 rpm for 10 minutes at 4°C. For immunoblotting experiments, 50 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% nonfat dry milk in TBS with 0.5% Tween 20 (Sigma). Antibodies used for different experiments were: polyclonals anti-c-IAP1 and anti-c-IAP2 from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-survivin (Abcam, Cambridge, United Kingdom); monoclonal anti-XIAP (BD Biosciences, San Jose, CA); polyclonal anti-Smac/DIABLO (Biomol International, Exeter, United Kingdom); monoclonal anti-Actin (Sigma). Appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Uppsala, Sweden) were added and proteins were detected using the enhanced chemiluminescence reagent (Amersham Biosciences).

For fractionation experiments, cytoplasmic proteins were obtained by lysing cells in hypotonic buffer (10 mmol/L Trizma Base [pH 8], 10 mmol/L KCl, 2 mmol/L phenylmethylsulfonyl fluoride, 2× Protease Inhibitor Cocktail, 0.2% NP40). After a 2 minute incubation in ice, cytosolic proteins were recovered by centrifugation at 2,000 rpm for 10 minutes at 4°C. Nuclear proteins were extracted resuspending the residual pellet in nuclear lysis buffer (10 mmol/L Trizma Base [pH 8], 10 mmol/L KCl, 100 mmol/L NaCl, 7 mmol/L β-Mercaptoethanol, 2× Protease Inhibitor Cocktail), and subsequently harvesting the proteins by centrifugation at 14,000 rpm for 15 minutes at 4°C.

The purity of the cytoplasmic and nuclear fractions was checked by Western blot analysis with monoclonal anti-Tubulin and polyclonal anti-Histone 2B antibodies (both from Santa Cruz).

Mitochondrial fractions were obtained using the ApoAlert Cell Fractionation Kit (BD Biosciences) following the manufacturer's protocol.

Immunofluorescence. Cells were fixed onto glass coverslips with 3.7% formaldehyde/PBS for 15 minutes, washed with PBS, permeabilized with 0.3% Triton X-100 for 5 minutes, washed again and incubated for 1 hour in 10% normal goat serum (Invitrogen). The coverslips were then incubated with anti-Smac polyclonal antibody and anti-cytochrome *c* monoclonal antibody (Upstate Cell Signaling, Lake Placid, NY) for 1 hour, followed by Alexa Fluor 594 rabbit anti-mouse and Alexa Fluor 488 goat anti-rabbit conjugates (Molecular Probes, Eugene, OR). After DNA staining with Hoechst 33258, coverslips were mounted onto glass slides with gel mount (Biomeda, Foster City, CA). Epifluorescence microscopy was done with an

Olympus microscope. The images were digitally acquired with an Orca CCD (Hamamatsu, Hamamatsu City, Japan) and processed with the Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Plasmids and transfections. To obtain a green fluorescent protein-tagged Smac/DIABLO, human Smac was amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the following primers:

forward 5′ -CTAGCTAGCATGGCGGCTCTGAAGAGTTG-3′;
reverse 5′ -CCGCTCGAGATCCTCACGCAGGTAGGCC-3′.

The expected 717 bp amplicon (full length Smac without the stop codon) was purified with the Wizard DNA purification kit (Promega, Madison, WI) and cloned in the *Nhe*I-*Xho*I restriction sites of the pEGFP-N1 expression vector (Clontech, Palo Alto, CA). Smac-GFP was then transfected in the indicated thyroid cancer cell lines with FuGENE 6 (Roche) according to the manufacturer's instruction.

Small interfering RNA experiments. WRO (3×10^5/well) and SW-1736 (4.5×10^5/well) cells were plated in multiwell plates and grown in medium without antibiotics for 24 hours. Cells at 90% to 95% confluency, were then transfected with small interfering RNAs (siRNA) for survivin, c-IAP1 or their scrambled counterpart (Dharmacon, Lafayette, CO) using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. The final concentration of the siRNAs was 100 nmol/L. For SW-1736 cells, a second transfection was carried out 24 hours after the first one to improve the silencing of c-IAP1. The efficacy of each siRNA was verified by Western blot analysis.

Statistical analysis. Unpaired, single-tail *t* tests with 95% confidence intervals were used to compare the expression of four IAPs and Smac in thyroid cancer cells before and after drug treatment. The level of each protein was determined by averaging the densitometric values of at least three independent experiments with expression in untreated cells arbitrarily set at 1. In silencing experiments, the same statistical approach was used to compare cell viability after drug treatment in cells transfected with siRNAs against the gene of interest versus cells transfected with a control scramble siRNA. Calculations were done using the PRISM software (GraphPad Software Inc., San Diego, CA).

Results

Thyroid cancer cells exhibit different sensitivity to chemotherapeutic agents. Treatment with antiblastic agents represents the best therapeutic option for patients affected by thyroid neoplasias that are insensitive to radioiodine treatment (12, 14). CDDP, doxorubicin, and taxol are among the cytotoxic drugs that have elicited the best response in patients affected by PDTCs and UTCs (17–19). However, the rapid occurrence of resistance leads to an often-fatal relapse of the disease.

To establish the efficacy of CDDP, doxorubicin, or taxol on human thyroid cancer cells, we incubated a panel of seven thyroid cancer lines with the three compounds for 24, 48, or 72 hours. At each time point, cell viability was assessed both by trypan blue exclusion and MTT assays (Fig. 1). After 24 hours of treatment with CDDP and doxorubicin, most thyroid cancer cells showed limited cell death (Fig. 1*A* and *B*). Longer exposure to the two drugs led to a substantial decrease in the number of viable cells. However, after being cultured for 72 hours in the presence of CDDP or doxorubicin, ~20% of thyroid cancer cells were still alive and metabolically active (Fig. 1*A* and *B*). Treatment with taxol induced a higher reduction of cell viability at all time points (Fig. 1*C*). Nevertheless, after a 72-hour incubation with taxol, >10% of each thyroid cancer line tested had not undergone death.

These results suggested that PDTCs and UTCs display different sensitivities to chemotherapeutic agents and that even prolonged exposure to high levels of these drugs may fail to eradicate the neoplastic population.

c-IAP1, c-IAP2, survivin, XIAP, and Smac are expressed in human thyroid cancer cells. In order to evaluate the possible contribution of different IAPs and their negative regulator Smac to the chemoresistance of PDTCs and UTCs, we needed to establish if these proteins were expressed in normal and neoplastic thyroid epithelial cells. Previously published evidence analyzing a single thyroid cancer cell line indicated that c-IAP1, XIAP, and Smac are expressed in human thyroid carcinomas (20, 21). More detailed analyses on human thyroid neoplasms have been carried out only for the expression of c-IAP2 and survivin (22, 23).

Hence, we did a detailed expression analysis on primary cultures derived from normal and neoplastic human thyrocytes, and on a panel of thyroid cancer cell lines. Normal thyrocytes expressed the mRNA for both c-IAP1 and c-IAP2 (Fig. 2*A, left*). We found a modest expression of the survivin transcript in one of the four normal specimens. Unexpectedly, we detected a low amount of mRNA for XIAP with a faint positivity in two of the four normal thyroid samples (Fig. 2*A, left*). On the contrary, Smac was highly expressed in all normal thyroid specimens. These data were substantially confirmed at the protein level with barely detectable levels of survivin and no XIAP expression (Fig. 2*B, left*).

When we repeated this analysis on primary cultures derived from iodine-insensitive thyroid carcinomas, we detected a strong transcript for c-IAP1 and c-IAP2 in three of six specimens, whereas survivin, XIAP, and Smac were clearly expressed in all cultures tested (Fig. 2*A, middle*). However, immunoblot experiments showed expression of the four IAPs and Smac in all six specimens (Fig. 2*B, middle*). Finally, thyroid cancer cell lines displayed high expression of the four IAPs and of Smac both at the mRNA and at the protein level (Fig. 2, *right*).

Thyroid cancer cells that resist chemotherapy-induced death express high levels of c-IAP1 and survivin but low levels of Smac. If thyroid cancer resistance to cell death triggered by chemotherapeutic drugs involves the IAP proteins, their expression should be increased in neoplastic cells that have not undergone death after antiblastic treatment. On the contrary, these cells should exhibit reduced levels of Smac, a negative regulator of IAP activity. To verify this hypothesis, we cultured seven thyroid cancer lines with CDDP, doxorubicin, or taxol for 48 hours and analyzed the expression of four IAPs and Smac in adhering cells that had survived treatment with the chemotherapeutic compounds.

Immunoblot experiments showed that thyroid cancer cells display different levels of IAPs and Smac in response to different chemotherapeutic drugs (Table 1). Specifically, c-IAP1 expression significantly increased after CDDP treatment in four of seven cell lines, whereas survivin was significantly higher in six of seven thyroid cancer lines (Fig. 3). In comparison, the expression of c-IAP2 and XIAP, after exposure to chemotherapeutic drugs, was not significant in the majority of the cancer cells investigated (Table 1; Fig. 3). When we measured the levels of Smac, we noticed infrequent reductions of the protein after treatment with CDDP or doxorubicin (Table 1). However, exposure to taxol led to the selection of cells that displayed reduced levels of Smac. This decrease was statistically significant in four of seven cell lines (Fig. 3). These data suggested that regulation of absolute levels of c-IAP1, survivin, and Smac may indeed be involved in the early resistance to chemotherapy observed in PDTC and UTC cells.

Thyroid cancer cells that survive treatment with chemotherapeutic agents partially retain Smac in the mitochondria. The intracellular localization of different IAPs regulates their biological activity and often correlates with the outcome of various

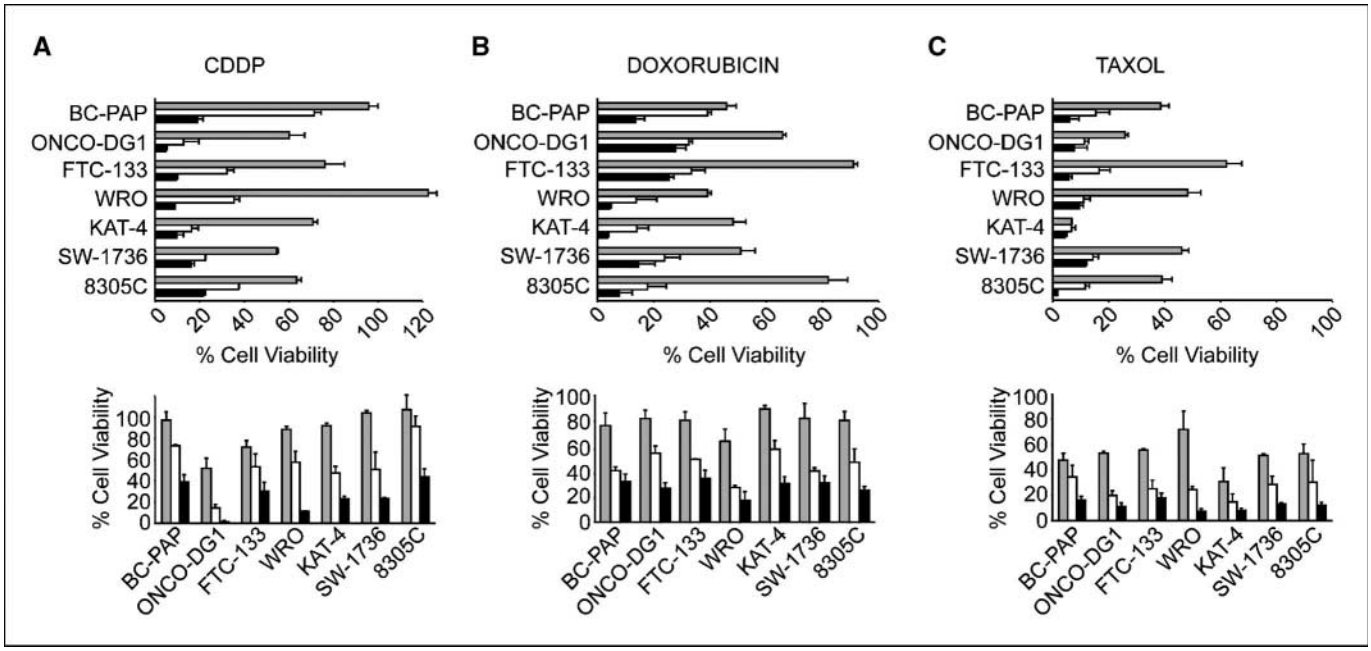

Figure 1. Cytotoxicity of different antiblastic compounds on thyroid cancer cells. BC-PAP, ONCO-DG1, FTC-133, WRO, KAT-4, SW-1736, and 8305C thyroid cancer cell lines were incubated with 5 μg/mL CDDP (*A*), 5 μmol/L doxorubicin (*B*), or 5 μmol/L taxol (*C*) for 24 (*gray columns*), 48 (*white columns*), or 72 (*black columns*) hours. For each time point, cell viability was assessed by trypan blue exclusion (*top*) or MTT assays (*bottom*). *Columns,* average of three independent experiments done in triplicate with untreated cells set at 100%; *bars,* ±SD.

forms of cancer (24–29). We therefore evaluated whether thyroid cancer cells that survived exposure to antiblastic compounds modified the subcellular localization of their c-IAP1, c-IAP2, survivin, and XIAP proteins.

Nuclear-cytoplasmic fractionation showed that c-IAP1 and c-IAP2 localized both to the nucleus and the cytoplasm of untreated BC-PAP and SW-1736 cells (Fig. 4*A*). However, c-IAP1 and c-IAP2 were almost exclusively cytosolic in WRO cells. CDDP and taxol did not affect the intracellular distribution of these IAPs (Fig. 4*A*). We found survivin expression only in cytoplasmic fractions of chemotherapy-naïve thyroid cancer cells, whereas XIAP was partially nuclear in the BC-PAP cell line and exclusively cytoplasmic in WRO and SW-1736 (Fig. 4*A*). Again, treatment with CDDP or taxol did not influence the intracellular localization of these proteins. Similar results were obtained when the cells were cultured in the presence of doxorubicin (data not shown).

We next sought to determine if Smac was being released from the mitochondria of thyroid cancer cells that had resisted antiblastic treatment. We initially did an immunofluorescence analysis on cells treated for 48 hours with CDDP and found very limited—if any—mitochondrial release of Smac (Fig. 4*B*). Indeed, in three cell lines, exposure to the drug caused a spatial

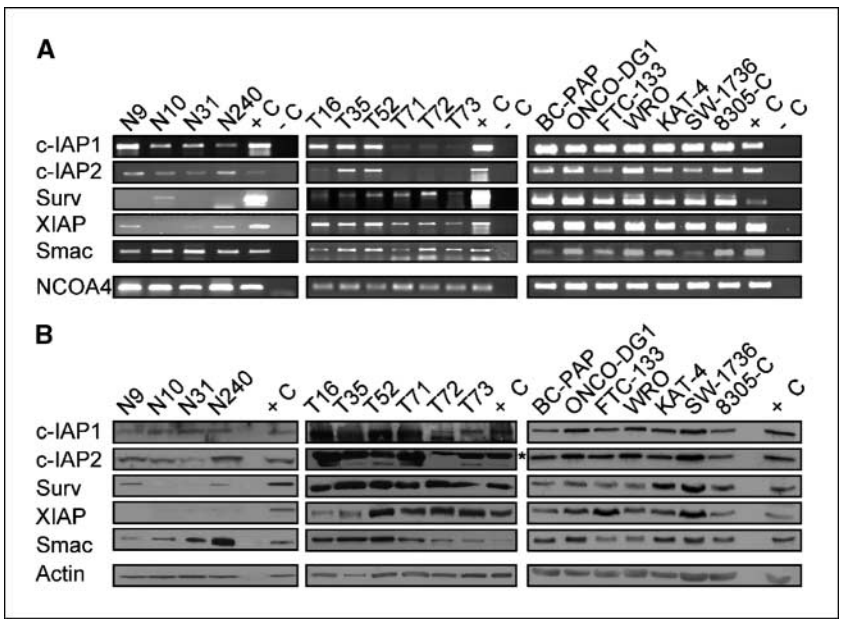

Figure 2. Expression of four IAPs and Smac in human normal or neoplastic thyrocytes, and thyroid cancer cell lines. RT-PCR (*A*) and Western blot (*B*) analysis were done on RNA or protein lysates derived from primary cultures of four normal human thyrocytes (*N*), six PDTCs (*T*) or the seven specified thyroid cancer lines. The U937 cell line was used as a positive control (+) whereas the absence of cDNA in the PCR reaction represented a negative control (−). Expression of the housekeeping gene *NCOA4* was used to verify the quality of the RNA extracted. **,* correct band for c-IAP2 in immunoblots done on PDTCs.

Table 1. Relative protein levels of different IAPs and Smac in thyroid cancer cells treated for 48 hours with the indicated chemotherapeutic drugs

Drug	BC-PAP	ONCO-DG1	FTC-133	WRO	KAT-4	SW-1736	8305-C	
CDDP	1.4 ± 0.35*	0.8 ± 0.3	1.1 ± 0.48	1.5 ± 0.27*	1.3 ± 0.19*	2.7 ± 0.58†	1.3 ± 0.24*	
DOX	1.3 ± 0.19*	1.2 ± 0.18	0.8 ± 0.56	0.9 ± 0.56	0.9 ± 0.52	1.8 ± 0.6*	1.3 ± 0.11*	c-IAP1
TAX	1.9 ± 0.47*	0.6 ± 0.26	1.8 ± 0.47*	1.3 ± 0.44	3.4 ± 0.17‡	1.5 ± 0.27*	1.7 ± 0.3†	
CDDP	2.2 ± 0.28‡	1.4 ± 0.53	0.5 ± 0.05	0.8 ± 0.32	0.8 ± 0.19	2.7 ± 0.93*	1.4 ± 0.05	
DOX	1.5 ± 0.47	1.4 ± 0.15†	0.4 ± 0.13	0.9 ± 0.20	0.8 ± 0.08	2.3 ± 1.74	1.3 ± 0.3†	c-IAP2
TAX	1.5 ± 0.12†	1.3 ± 0.29	1.0 ± 0.26	0.5 ± 0.14	1.7 ± 0.25*	1.2 ± 0.6	1.0 ± 0.1	
CDDP	0.9 ± 0.04	1.4 ± 0.05†	2.1 ± 0.28‡	0.8 ± 0.49	2.1 ± 0.67*	1.7 ± 0.56*	1.4 ± 0.3*	
DOX	3.1 ± 0.24‡	1.8 ± 0.85	2.7 ± 0.46†	3.6 ± 0.51‡	2.1 ± 0.72*	1.7 ± 0.45*	1.8 ± 0.28†	Surv
TAX	6.4 ± 0.6‡	1.9 ± 0.65*	0.7 ± 0.32	0.8 ± 0.53	2.7 ± 0.09‡	1.7 ± 1.08	1.9 ± 0.73*	
CDDP	1.4 ± 0.08†	1.9 ± 0.57	1.2 ± 1.27	0.8 ± 0.26	0.8 ± 0.34	0.2 ± 0.37	1.8 ± 0.63*	
DOX	6.0 ± 1.67†	1.2 ± 0.32	2.1 ± 0.81*	0.7 ± 0.18*	0.8 ± 0.39	1.8 ± 0.54*	0.3 ± 0.07	XIAP
TAX	3.2 ± 1.3*	0.9 ± 0.05	0.9 ± 0.33	1.3 ± 0.45	1.2 ± 0.79	1.9 ± 0.53*	0.5 ± 0.32	
CDDP	1.1 ± 0.5	0.8 ± 0.47	1.5 ± 0.21	0.8 ± 0.1*	0.7 ± 0.48	1.8 ± 0.06	0.5 ± 0.03†	
DOX	1.6 ± 0.37	1.0 ± 0.02	0.6 ± 0.02†	0.9 ± 0.12	0.8 ± 0.14	1.7 ± 0.29	1.2 ± 0.18	Smac
TAX	0.5 ± 0.28†	0.6 ± 0.15*	0.6 ± 0.07†	1.0 ± 0.4	0.6 ± 0.35	0.5 ± 0.38	0.6 ± 0.04†	

NOTE: Statistical analysis indicates significance of increases in IAPs expression or reduction of Smac. Mean ± SD of three independent experiments with expression in untreated cells = 1.

*$P < 0.05$.

†$P < 0.01$.

‡$P < 0.001$.

rearrangement of the mitochondrial figures. However, we did not observe any modification in the mitochondrial localization of either Smac or cytochrome *c* (Fig. 4*B*). These data were further validated by mitochondrial fractionation experiments, showing a modest cytoplasmic relocalization of cytochrome *c* after treatment with CDDP, doxorubicin, and taxol and an even narrower mitochondrial displacement of Smac (Fig. 4*C*).

Our results indicated that mitochondrial retention of Smac might also be involved in the lack of cell death that is observed after exposure of thyroid cancer cells to various chemotherapeutic compounds.

Silencing of c-IAP1 and survivin and overexpression of Smac increase the efficacy of chemotherapy on thyroid cancer cells. Our previous results indicated that overexpression of c-IAP1 and survivin is frequently observed in thyroid cancer cells that survive 2 days of treatment with CDDP and doxorubicin (Fig. 3). Moreover, thyroid cancer lines that are not killed by taxol display reduced levels of Smac and partially retain this IAP inhibitor in the mitochondria (Figs. 3 and 4). In order to establish if these changes were directly related to the ability of thyroid cancer cells to resist the damage inflicted by antiblastic agents, we did two sets of experiments.

We first silenced the expression of c-IAP1 and survivin in SW-1736 and WRO cells, respectively. Immunoblot analysis confirmed that transfection of these cells with the specific siRNAs (but not with scrambled controls, siSCR) resulted in a strong reduction of each IAP (Fig. 5*A* and *B, left*). When SW-1736 cells with reduced levels of c-IAP1 were exposed to CDDP for 24 hours, >95% of the cells underwent apoptosis (Fig. 5*A, top right*). This result was in stark contrast with the amount of death (60%) observed in cells transfected with the scrambled siRNA, which was comparable with the death rate detected by trypan blue exclusion in untransfected SW-1736 cells treated with CDDP (Fig. 1*A, top*). When CDDP treatment was prolonged for 48 hours, >99% of cells with silenced c-IAP1 were killed by CDDP (Fig. 5*A, right*). This was not the case with cells transfected with control siSCR that presented an 80% death rate, superimposable to that observed in SW-1736 cells exposed to CDDP (Fig. 1*A, top*). Similarly, WRO cells with reduced expression of survivin displayed a statistically significant increase in the amount of cell death after doxorubicin treatment for 24 and 48 hours (Fig. 5*B, right*). Again, this result was not observed in cells transfected with the control siSCR.

In a second series of experiments, we transfected GFP-tagged Smac in BC-PAP, WRO and 8305C cells that were subsequently treated with taxol. After a 48-hour exposure to the drug, cells overexpressing Smac had a significantly higher mortality rate than cells transfected with GFP-tagged histone 2B as a control (Fig. 5*C*). Our results indicated that increased levels of c-IAP1 and survivin or the reduced expression of Smac are directly involved in the early resistance to death stimuli exhibited by PDTC and UTC cells after exposure to chemotherapeutic drugs.

Thyroid cancer cells that have acquired permanent resistance to antiblastic compounds require high levels of c-IAP1 or survivin. We next wanted to determine if thyroid cancer cells that had become permanently resistant to different chemotherapeutic agents would also express high levels of c-IAP1 and survivin and reduced levels of Smac. Thus, we obtained clones of WRO cells that were able to proliferate in the presence of CDDP, doxorubicin, or taxol and analyzed them for the expression of the aforementioned proteins (Fig. 6*A*).

WRO cells that were permanently resistant to CDDP (WRO R-CDDP) displayed a 5-fold increase in the expression of survivin (Fig. 6*B, top*), but showed the same levels of c-IAP1 of their sensitive counterpart and no decrease in Smac expression. WRO cells that had become permanently resistant to doxorubicin (WRO R-DOX), presented a 5-fold increase in the expression of c-IAP1 (Fig. 6*B, middle*) but showed minimal changes in the expression of

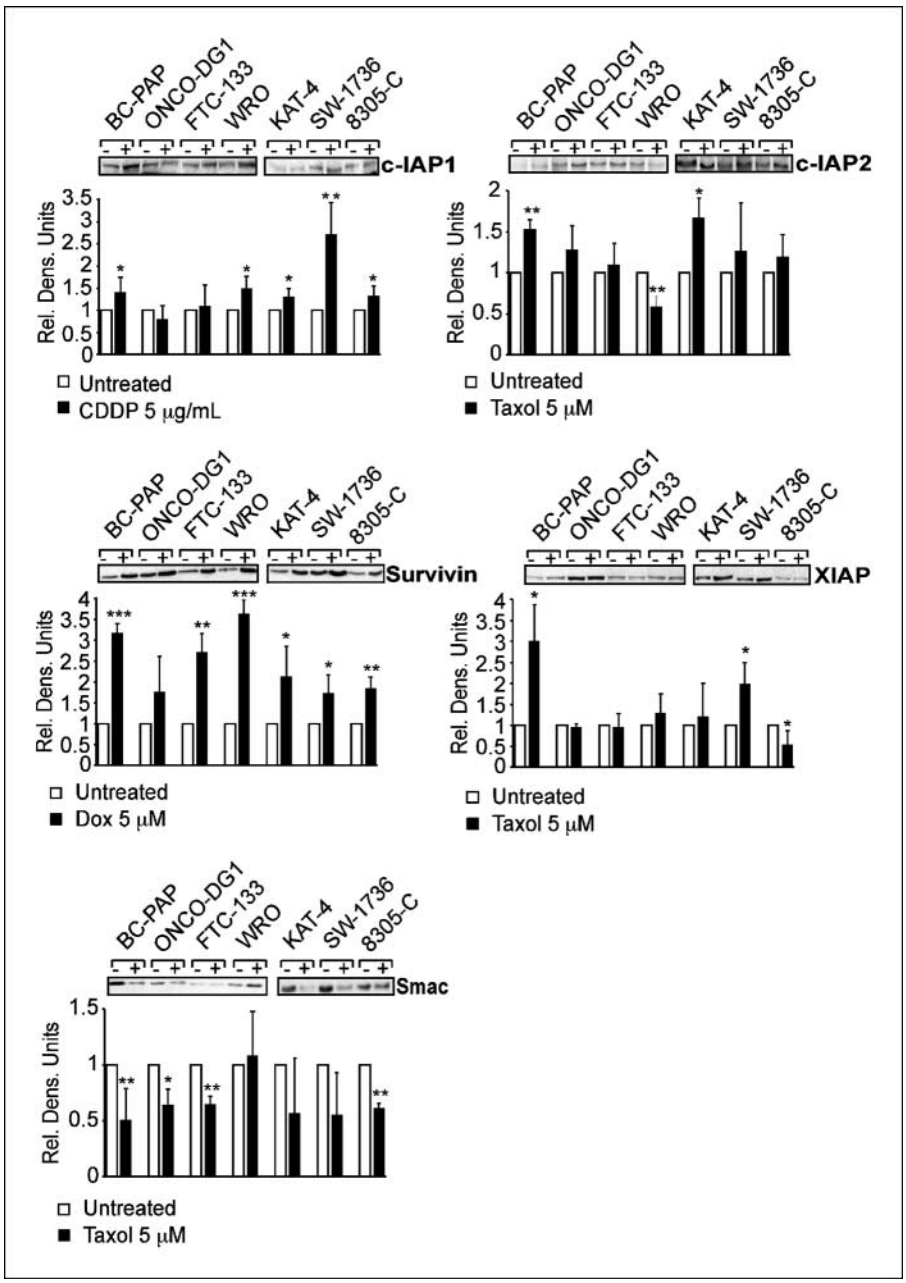

Figure 3. Different chemotherapeutic compounds select thyroid cancer cells with high c-IAP1 and survivin and low Smac. Thyroid cancer cells were either left untreated (−) or exposed for 48 hours to the specified compounds (+). Cell lysates extracted from each experimental condition were then blotted with antibodies against c-IAP1, c-IAP2, survivin, XIAP, and Smac. *Columns,* average of densitometric analyses of three individual experiments; *bars,* ±SD (*bottom*). The expression of each protein in untreated cells (*white columns*) was arbitrarily set at 1 and compared with the level found in cells treated with the indicated compounds (*black columns*). *, $P < 0.05$; **, $P < 0.01$.

survivin and Smac. These results were partially unexpected because we had not seen such changes in WRO cells that had survived a 48-hour incubation with either CDDP or doxorubicin (Table 1). WRO clones that had acquired resistance to taxol showed no differences in the levels of c-IAP1, survivin, or Smac when compared with taxol-sensitive cells (Fig. 6*B, bottom*).

To further show that IAP overexpression contributes to the acquisition of resistance to chemotherapy, we silenced survivin expression in WRO R-CDDP cells and analyzed their response to CDDP (Fig. 6*C*). Treatment with the drug for 24 hours induced minimal death in cells transfected with a scramble control but killed 53% of the previously resistant cells that had been transduced with anti-survivin siRNA (Fig. 6*C*). The amount of dead cells increased to 80% after 48 hours of CDDP exposure. Likewise, when we silenced c-IAP1 in WRO R-DOX cells and subsequently exposed them to doxorubicin, we observed an 80% death rate after 24 hours, which increased to 90% at 48 hours (Fig. 6*D*). No such results were detected in WRO R-DOX cells transfected with control scramble siRNAs.

These data strongly implied that high expression levels of different IAPs are directly involved in PDTC and UTC resistance to chemotherapy and that silencing of overexpressed IAPs can restore cell sensitivity to chemotherapeutic agents.

Discussion

Carcinomas of the thyroid of follicular origin are usually treated with surgical removal of the gland and, if necessary, radioablative treatment to eradicate possible neoplastic remnants (10). However, ~10% of patients affected by thyroid cancer will present with

completely undifferentiated phenotypes or will display relapsed cancerous lesions that have acquired poorly differentiated pathologic characteristics (12, 13). These tumors have lost both the morphologic and functional characteristics of a differentiated thyroid gland and fail to uptake and retain radioactive iodine. For these individuals, treatment with different combinations of chemotherapeutic agents presently represents the most effective therapeutic approach. Unfortunately, the majority of these tumors will present or eventually develop resistance to chemotherapy and progress to a negative outcome.

Well-characterized mechanisms of chemotherapeutic resistance include alterations of the specific target for an antiblastic agent, defects in the transport of the drug or increases of its metabolic clearance (15, 30). However, it is becoming increasingly clear that a crucial variable for the development of chemoresistance lies in the ability of neoplastic cells to survive the initial death stimuli triggered by exposure to antiblastic agents (16). In this regard, research on the mechanisms responsible for chemoresistance has been recently focused on molecules involved in the regulation of cell death (31).

We report here that c-IAP1, survivin, and their negative regulator, Smac, are directly involved in the development of chemoresistance by PDTC and UTC cells. Short exposure (48 hours) of thyroid cancer cells to CDDP, doxorubicin, or taxol showed that these tumor cells could overcome unfavorable environmental conditions when overexpressing c-IAP1, survivin or when exhibiting low levels of Smac. Moreover, thyroid cancer cells that survive exposure to chemotherapy display very limited—if any—mitochondrial release of Smac. Silencing of c-IAP1 or survivin promptly increases the death rates induced by CDDP or doxorubicin. Likewise, overexpression of Smac significantly augments the cytotoxic effect of taxol. Furthermore, thyroid cancer cell lines that have acquired permanent resistance to different chemotherapeutic drugs and actively proliferate when cultured in the presence of antiblastic compounds express high levels of different IAPs. Specifically, CDDP resistant cells express high levels of survivin, whereas cells resistant to doxorubicin overexpress c-IAP1. Silencing of these IAPs by siRNA restored cell sensitivity to chemotherapeutic agents.

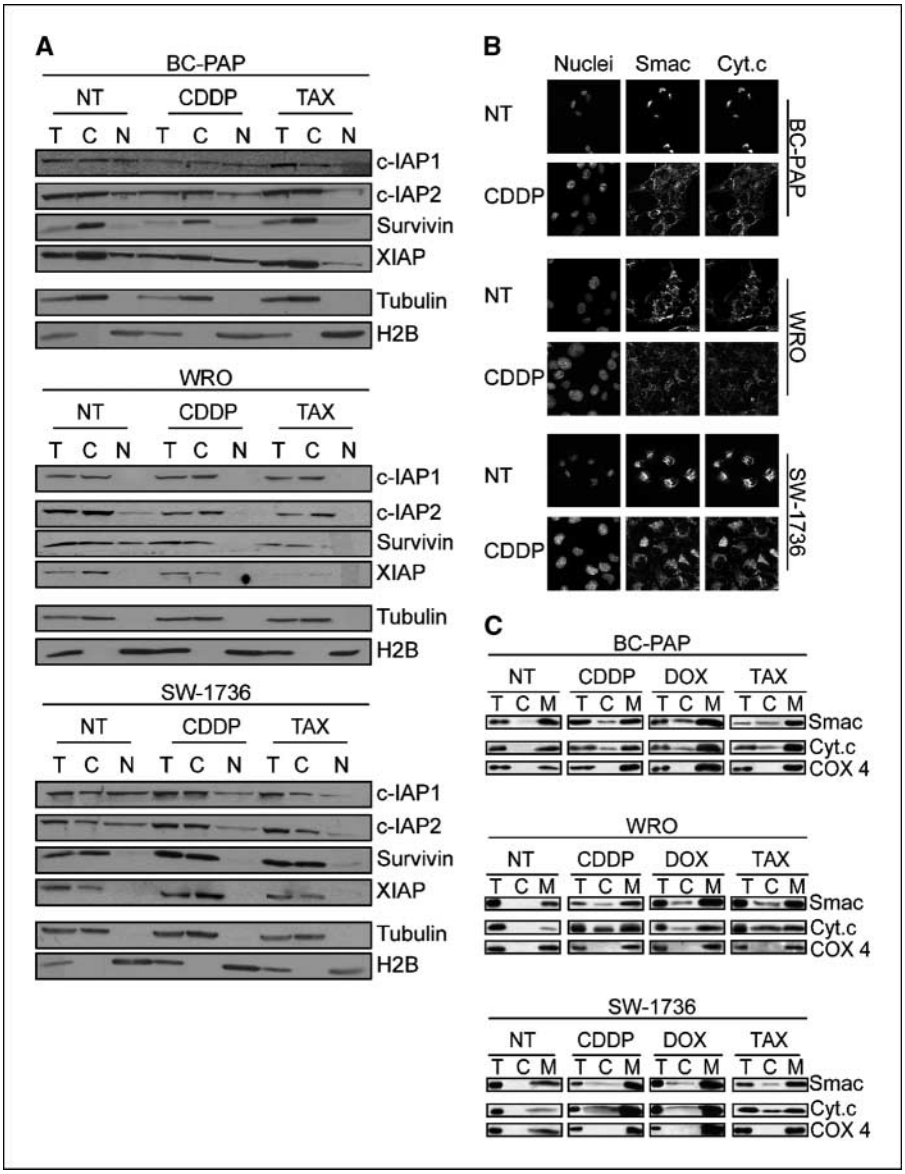

Figure 4. Exposure to chemotherapeutic agents does not cause mitochondrial release of Smac in thyroid cancer cells. *A,* total (*T*), cytoplasmic (*C*), and nuclear (*N*) lysates were isolated from BC-PAP, WRO, and SW-1736 thyroid cancer cells exposed for 48 hours to 5 μg/mL CDDP or 5 μmol/L taxol. The different fractions were then immunoblotted with antibodies against the indicated proteins. Western blots for tubulin and histone 2B confirmed the purity of the cytoplasmic and nuclear extracts. *B,* the same cell lines were stained to visualize the nuclei, Smac, and cytochrome *c* before (*NT*) and after treatment for 48 hours with 5 μg/mL CDDP. *C,* the indicated cells were either left untreated (*NT*) or exposed for 48 hours to 5 μg/mL CDDP, 5 μmol/L doxorubicin, or 5 μmol/L taxol. Cells were then subjected to mitochondrial fractionation and total (*T*), cytoplasmic (*C*), or mitochondrial (*M*) lysates were probed with an anti-Smac antibody. Immunoblots for cytochrome *c* and cyclooxygenase-4 were carried out to verify the quality of the mitochondrial fractions.

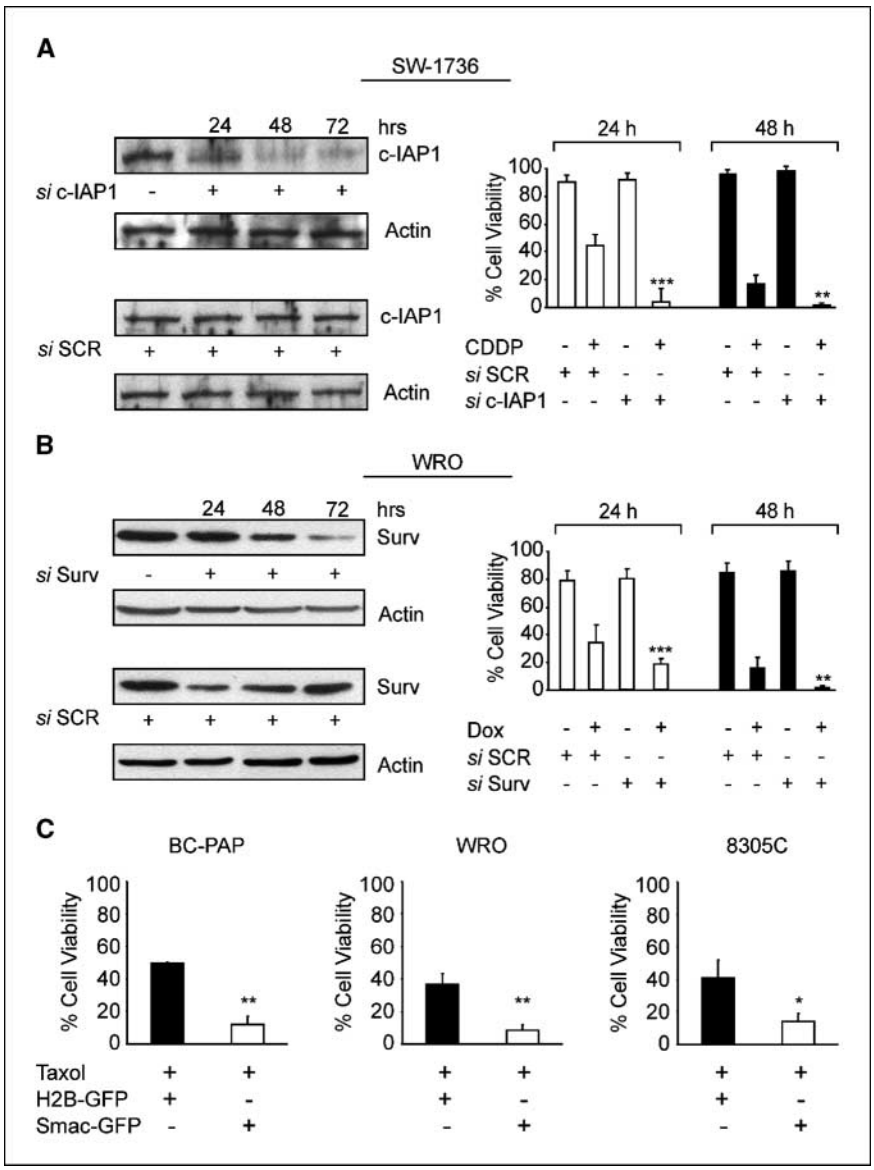

Figure 5. Silencing of c-IAP1 and survivin or overexpression of Smac increase thyroid cancer cell response to chemotherapy. *A,* SW-1736 thyroid cancer cells were transfected with siRNAs for c-IAP1 (*left*), or their scrambled equivalent (*si SCR*). After 24, 48, or 72 hours, cells were analyzed by Western blotting for their expression of c-IAP1. Twenty-four hours after siRNA transfection, SW-1736 cells were either left in normal media or treated with 5 μg/mL CDDP. Cell viability was then assessed by trypan blue exclusion in each experimental condition after 24 or 48 hours of drug treatment (48 or 72 hours after siRNA transfection). Results shown correspond to the average ± SD of three separate experiments done in duplicates. *B,* the same experiment was repeated using WRO cells that were transfected with siRNA for survivin. Twenty-four hours later, cells were either left untreated or exposed to 5 μmol/L doxorubicin for 24 or 48 hours. *C,* BC-PAP, WRO, and 8305C cells were transiently transfected with Smac-GFP or histone 2B-GFP (as a control) and, 24 hours later, treated with 5 μmol/L of taxol for 2 days. Cells were then fixed, stained with Hoechst to visualize their nuclear compartment, and the percentage of apoptotic cells was calculated evaluating the number of condensed nuclei. *Columns,* mean of four experiments with 3,000 cells counted for each condition; *bars,* ±SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Our results raise several intriguing questions about the exact role of IAPs and Smac in thyroid cancer resistance to chemotherapy.

The first issue concerns the mechanisms underlying the high levels of c-IAP1 and survivin and the low levels of Smac in cells that survive brief exposure (48 hours) to chemotherapy. Are the changes in the expression of these proteins caused by transcriptional induction (c-IAP1 and survivin) or repression (Smac) in response to chemotherapy, or are we simply registering a selection process in which cells that express higher levels of IAPs or lower levels of Smac naturally benefit from their genomic asset? Preliminary experiments done by real-time reverse transcription-PCR have shown no increase in the transcripts for c-IAP1 and survivin after exposure to CDDP or doxorubicin (data not shown). On the contrary, treatment with taxol seems to down-regulate the transcript for Smac (data not shown). Hence, a selection mechanism might be more likely in the case of the two IAPs, whereas a transcription-dependent mechanism could be responsible for the decreased expression of Smac after taxol treatment.

A second issue concerns the surprising evidence pointing to a direct role of some IAP proteins in the permanent development of chemoresistance. The fact that c-IAP1 or survivin are highly expressed in WRO cells resistant to CDDP or doxorubicin, respectively, strongly implies that, in this cell context, the role of IAP proteins is not limited to the initial abrogation of death necessary to escape drug cytotoxicity. Instead, these data unveil an unexpected scenario in which acquisition of permanent resistance to chemotherapy by thyroid cancer cells is a multistep process that requires: (*a*) early suppression of cell death after exposure to antiblastic compounds, (*b*) the use of this survival window to select one or more mechanisms that will allow the neoplastic cells to permanently evade the effect of chemotherapeutic agents, (*c*) the restoration of proper replicative and/or metabolic activities while stably increasing the death threshold. Our results indicate that IAP proteins and Smac can be involved in the first step although only IAPs play a role in the last part of the process. Further experiments will be necessary to determine if IAPs and Smac are also involved in the second (and more critical) step of this process.

A third issue regards the surprising finding that exposure of thyroid cancer cells to the same chemotherapeutic agent for different time frames results in the increased expression of different IAPs. Indeed, when we treated WRO cells for 48 hours with CDDP, we observed increased expression of c-IAP1, but no differences in survivin expression (Table 1). However, WRO cells that are permanently resistant to CDDP show a strong increase in survivin expression and minor changes in c-IAP1 levels (Fig. 6*B*). This result was not dependent on the specific cell line used because SW-1736 cells that have become resistant to CDDP also display high levels of survivin and no increase in c-IAP1 expression (data not shown). In addition, WRO cells that have acquired

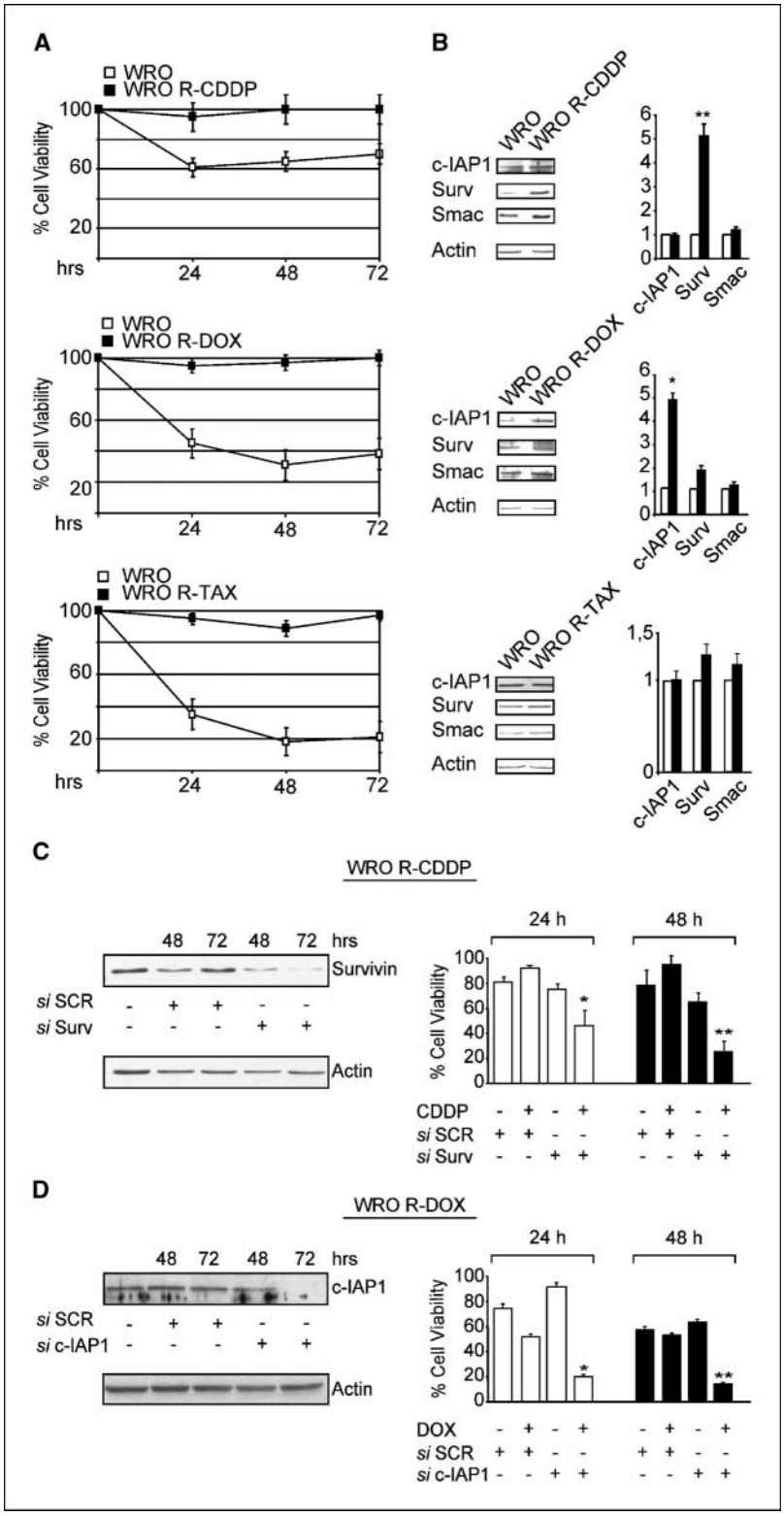

Figure 6. Thyroid cancer cells permanently resistant to chemotherapeutic agents express high levels of c-IAP1 or survivin and silencing of these IAPs restores their response to antiblastic compounds. *A,* WRO cells were grown in the continuous presence of increasing amounts of CDDP, doxorubicin, or taxol until they acquired permanent resistance to the cytotoxic effects of the drugs. Sensitive (□) or drug-resistant cells (■) were then exposed to the indicated compounds for 24, 48, or 72 hours and cell viability was assessed by trypan blue exclusion. *Points,* average of three separate experiments done in triplicate; *bars,* ±SD. *B,* protein extracts derived from sensitive or resistant cells were immunoblotted with antibodies against c-IAP1, survivin, or Smac. *Columns,* average fold change of the indicated protein from three separate experiments; *bars,* ± SD (*right*). Expression levels in sensitive cells were arbitrarily set at 1. *C,* WRO cells resistant to CDDP or to doxorubicin (*D*) were transfected with siRNAs for survivin (*si Surv*), c-IAP1 (*si c-IAP1*) or the corresponding scrambled sequences (*si SCR*), and analyzed by Western blot to confirm the silencing of the two IAPs. Twenty-four hours after transfection, cells were treated with 5 μg/mL CDDP or 1 μmol/L doxorubicin and their viability was determined by trypan blue exclusion. *Points,* mean of three separate experiments done in duplicate; *bars,* ±SD; *, $P < 0.05$; **, $P < 0.01$.

resistance to doxorubicin present high levels of c-IAP1 but no increase in survivin expression (Fig. 6*B*). Again, this pattern diverges from the one observed after brief exposure of the cells to doxorubicin, where we observed high levels of survivin and no increase in c-IAP1 (Table 1). Although we do not have a mechanistic explanation for these findings, the data effectively implies that when PDTC and UTC cells progress through the sequential steps leading to the development of chemoresistance, they require high levels of different IAPs. In other terms, in order to survive the initial exposure to CDDP, WRO cells necessitate high amounts of c-IAP1, whereas high expression of survivin is required to resist the deleterious effects of doxorubicin. However, after acquiring escape mechanisms that allow them to proliferate in the continuous presence of the drugs, thyroid cancer cells need high levels of different IAPs. That survivin and c-IAP1 play a pivotal role in WRO R-CDDP and WRO R-DOX survival is confirmed by siRNA experiments showing that knockdown of the proteins promptly restores cell sensitivity to CDDP and doxorubicin (Fig. 6*C* and *D*).

Increasing evidence point to IAPs, and their negative regulator, Smac, as attractive novel targets for cancer therapy (32, 33). Indeed, several reports have suggested that high IAP expression is involved in the development or the progression of different tumors (34–40). Our data identify c-IAP1, survivin, and Smac as proteins actively implicated in thyroid cancer resistance to cell death triggered by CDDP, doxorubicin, and taxol. Furthermore, our findings also suggest that c-IAP1 and survivin are directly involved in the acquisition of permanent chemotherapy resistance by thyroid cancer cells. Taken together, these results identify c-IAP1 and survivin as potential targets for therapeutic interventions aimed at restoring the sensitivity of PDTC and UTC cells to chemotherapy. Targeting of these two IAPs by RNA interference, immunologic approaches (monoclonal antibodies), short polypeptides that negatively regulate their function, or by blocking their downstream biological signaling could potentially become a viable therapeutic approach for patients diagnosed with PDTCs or UTCs that are unresponsive to chemotherapeutic agents.

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Altered Expression of c-IAP1, Survivin, and Smac Contributes to Chemotherapy Resistance in Thyroid Cancer Cells

Elena Tirrò, Maria Letizia Consoli, Michele Massimino, et al.

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