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Drug specific resistance to oxaliplatin is associated with apoptosis defect in a cellular model of colon carcinoma

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Abstract To investigate acquired resistance to oxaliplatin, we selected two resistant clones from the HCT116 cell line. We found that the resistant phenotype was associated with resistance to oxaliplatin-induced apoptosis as demonstrated by FACS analysis and by Western blotting of caspase 3 activation. In addition, the resistant phenotype showed a concomitant resistance to lonidamine and arsenic trioxide which are inducers of mitochondrial apoptosis. Furthermore, a complete loss of Bax expression due to a frameshift mutation was observed in the most resistant clone. Taken together, these findings suggest that altered mitochondrial-mediated apoptosis could play a role in oxaliplatin resistance.

Key words: Oxaliplatin; Drug resistance; Apoptosis; Bax; Colorectal cancer

1. Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in Western countries [1,2]. The number of new cases of colorectal cancer worldwide is increasing, and approximately one half of colorectal cancer patients develop metastatic disease. The prognosis for these patients is poor because of frequent chemoresistance which is either intrinsic or acquired during treatment.

For a long time, 5-fluorouracil (5-FU) was the only drug used in advanced colorectal cancer until two novel chemotherapeutic agents, oxaliplatin and irinotecan in conjunction with 5-FU, were introduced and shown to have a clinical benefit [3,4]. In particular, oxaliplatin is a third generation platinum complex with a wide spectrum of anticancer activity. Oxaliplatin in association with 5-FU is active in patients with 5-FU-resistant disease (response rates up to 30–40% after 5-FU treatment failure) [5].

Resistance to conventional platinum derivatives such as cisplatin has been extensively studied and was shown to result from several mechanisms, including decreased drug transport, increased cytoplasmic detoxification by glutathione or metallothioneins, enhanced DNA repair and defect in apoptosis [6]. Although oxaliplatin produces the same type of DNA cross-links as cisplatin [7], differences still exist between the two agents as shown by the fact that cisplatin-resistant cells generally remain sensitive to oxaliplatin. DNA repair-related resistance to cisplatin can be due to an increase in nucleotide excision repair or to a defect in DNA mismatch repair (MMR), resulting from replicative by-pass of platinum adducts [8,9]. The same defects in MMR or replicative bypass, however, do not confer resistance to oxaliplatin [10,11].

Only two studies have reported the identification of a potential molecular mechanism for resistance to oxaliplatin. The first described the increase of γ-glutamyl transpeptidase activity leading to elevation in cellular GSH in sublines derived from A2780 ovarian carcinoma cell line [12]. Based on the same cellular model, a second study demonstrated reduced platinum accumulation and DNA–platinum adduct levels associated with oxaliplatin resistance [13]. The paucity of information relative to oxaliplatin resistance underlines the fact that a better understanding of the molecular events governing oxaliplatin activity and resistance is critical for its rational use.

To improve the knowledge of the molecular basis of resistance to oxaliplatin, we generated an in vitro cellular model of resistance derived from the HCT116 colon carcinoma cell line. The acquired resistance to oxaliplatin was found to be associated with resistance to oxaliplatin-induced apoptosis and with a concomitant resistance to lonidamine-induced apoptosis, suggesting a defect in the mitochondrial apoptotic pathway. Additionally, a loss of Bax expression was found in the most resistant clone (HCT116/R2), supporting the idea that Bax could represent one of the altered factors contributing to an apoptotic defect in oxaliplatin-resistant cells.

2. Materials and methods

2.1. Cell culture

Three clones (HCT116/S, HCT116/R1 and HCT116/R2) were derived from the HCT116 cell line (ATCC, Manassas, VA, USA) and were grown in RPMI 1640 medium supplemented with 10% fetal

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Abbreviations: 5-FU, 5-fluorouracil; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MMR, mismatch repair

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bovine serum and 2 mM glutamine without antibiotics. HCT116/R1 and HCT116/R2 cell lines were selected by exposure to stepwise increased concentrations of oxaliplatin and were maintained in the presence of 5 μM and 10 μM oxaliplatin, respectively. Before each experiment, HCT116/R1 and HCT116/R2 cells were cultured without oxaliplatin for 1 week.

2.2. Drug sensitivity assay

Growth inhibition assays were performed by seeding 4000 cells per well in 96-well microtiter plates. After incubation for 24 h for cell attachment, the drugs were added and the cells were incubated again for 48 h. Each drug concentration was tested in triplicate. Cytotoxicity was measured by the WST-1 colorimetric assay (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. Absorbance was measured at 450 nm 3 h after addition of the WST-1 substrate. The results were expressed in terms of the concentration of drug required to inhibit cell growth by 50% relative to untreated cells (IC_{50}).

2.3. Detection of apoptotic cells by flow cytometry

The cells were treated with 15 μM oxaliplatin or 35 μM irinotecan for 48 h and with 220 μM lonidamine or 40 μM arsenic trioxide for 24 h. For oxaliplatin, irinotecan and lonidamine treatments, the cells were washed and labeled using the fluorescein isothiocyanate (FITC)-labeled annexin V method (FITC-annexin V, Boehringer Mannheim)[14] according to the manufacturer's recommendations. For arsenic trioxide treatment, the cells were washed and incubated with the MitoCapture reagent (BioVision, Mountain View, CA, USA) according to the manufacturer's recommendations. After labeling, apoptotic cells were analyzed with a FACScan fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, MD, USA) using the FITC channel.

2.4. Western blotting analysis

Subconfluent monolayers of cells were washed twice in phosphate-buffered saline (PBS; pH 7.4, 135 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 6.38 mM Na_2HPO_4) and lysed in SDS buffer (bromophenol blue, 0.7 M 2-mercaptoethanol, 2% SDS, 10% glycerol and 0.5 M Tris–HCl). The extracts were sonicated and then boiled for 5 min. 100 000 cells per lane were separated by 12% SDS–PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting. Blots were blocked 50 min at room temperature in PBS–0.1% Tween 20 containing 5% non-fat dried milk, incubated for 2 h at room temperature with primary antibody [anti-Bax mouse monoclonal antibody (Neomarkers, Fremont, CA, USA), anti-Bak mouse monoclonal antibody (Oncogene, Boston, MA, USA), anti-Bcl2 mouse monoclonal antibody (Neomarkers), anti-Bcl-X_L mouse monoclonal (PharMingen, San Diego, CA, USA), anti-procaspase 3 mouse monoclonal antibody (BD Biosciences, Palo Alto, CA, USA) or rabbit anti-caspase 3 antibody (Cell Signaling, Beverly, MA, USA)] and then washed with PBS–0.1% Tween 20. Blots were then incubated for 1 h at room temperature in blocking buffer with the corresponding peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG antibody (Sigma, St. Louis, MO, USA) and washed again. Bound complexes were detected using the ECL-Plus reagent (Amersham Biosciences, France) according to the manufacturer's recommendations. To confirm equivalent loading and transfer of proteins, anti-α-tubulin antibody (Sigma) was used.

2.5. Amplification of the Bax G8 tract region for sequencing

Genomic DNA from each variant derived from the HCT116 cell line (HCT116/S, HCT116/R1 and HCT116/R2) was isolated. A 94-bp region encompassing the Bax G8 tract spanning codons 38–41 was amplified by PCR using the 5′-ATC CAG GAT CGA GCA GGG CG-3′ and 5′-ACT CGC TCA GCT TCT TGG TG-3′ primers as described by Ionov et al.[15]. PCR was carried out with Taq polymerase (MBI Fermentas, Vilnius, Lithuania) for one cycle at 94°C followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. PCR products were purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, France) before sequencing.

3. Results

3.1. Establishment of a cellular model for oxaliplatin resistance

To compare sensitive and resistant phenotypes in the context of the same genetic background, we derived several sublines from the human colorectal cell line HCT116. This cell line was previously found to be sensitive to oxaliplatin in the National Cancer Institute's Anticancer Drug screening panel [16]. The homogeneity of the HCT116 cells was verified by isolating different clones and determining their sensitivity to oxaliplatin: the IC_{50} values for these clones ranged from 0.3 to 1 μM (data not shown). One clone, referred to as HCT116/S, was selected for the present study and used as the sensitive variant. In parallel, after the HCT116 cell line had been continuously grown in the presence of stepwise increasing concentrations of oxaliplatin (from 0.1 μM to 10 μM), two resistant clones (HCT116/R1 and HCT116/R2) were isolated. HCT116/R1 and HCT116/R2 are 28- and 68-fold more resistant than HCT116/S, respectively (Table 1).

To evaluate the specificity of the resistance to oxaliplatin, we tested the sensitivity of the two oxaliplatin-resistant clones to cisplatin and irinotecan. Cisplatin was selected because it is related to oxaliplatin. Oxaliplatin and cisplatin generate similar inter- and intra-strand DNA cross-links. However, the DNA–platinum adducts produced by oxaliplatin are less accessible to DNA repair mechanisms, probably due to the presence of its bulky diaminocyclohexane group. Cisplatin did not demonstrate efficacy in the treatment of colorectal cancers. The cross-resistance to irinotecan was also assessed because this drug, an efficient inhibitor of DNA topoisomerase I, was recently introduced for the treatment of colorectal cancers. Results presented in Table 1 show that a low cross-resistance to irinotecan was observed in HCT116/R1. Cross-resistance in this model was not correlated, however, with the oxaliplatin resistance level since there was no cross-resistance to irinotecan in HCT116/R2. For cisplatin, we observed minimal cross-resistance in both HCT116/R1 and HCT116/R2 (2.1- and 2.9-fold, respectively). This low level of cross-resistance to cisplatin confirmed the specificity of the resistance to oxaliplatin in our model.

Table 1
Sensitivity of the oxaliplatin-resistant cell lines to cisplatin and irinotecan

HCT116 clone	IC_{50} (μM)[a]		
	Oxaliplatin	Cisplatin	Irinotecan
HCT116/S	0.32 ± 0.08 (1.0)[b]	4.7 ± 1.8 (1.0)	7.7 ± 3.8 (1.0)
HCT116/R1	8.9 ± 3.6 (27.8)	9.7 ± 2 (2.1)	32.3 ± 5.1 (4.2)
HCT116/R2	21.9 ± 6.3 (68.4)	13.4 ± 6.6 (2.9)	9.5 ± 4.2 (1.2)

[a]IC_{50} values were measured by the WST-1 colorimetric assay after continuous exposure to drug for 48 h. Values are means ± S.D. of data obtained from at least three independent experiments.
[b]Numbers in parentheses are the relative resistance determined as the ratio of the IC_{50} for the clone divided by the IC_{50} for the sensitive HCT116/S cells.

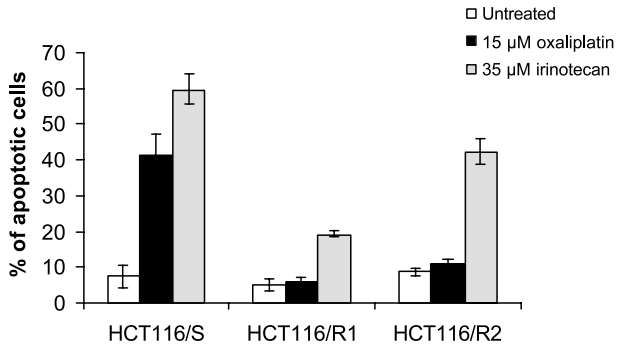

Fig. 1. Evaluation of oxaliplatin- or irinotecan-induced apoptosis in HCT116-derived sensitive and resistant clones. Apoptosis was evaluated 48 h after the beginning of treatment. The percentage of cells entering apoptosis was determined by FACS analysis using FITC-labeled annexin V. Results are the means of three independent experiments; bars, S.D. Cells were treated for 48 h with 15 μM oxaliplatin or 35 μM irinotecan.

3.2. *Oxaliplatin-resistant phenotypes are associated with a specific resistance to oxaliplatin-induced apoptosis*

As the mechanisms controlling oxaliplatin resistance are still unknown, we performed a preliminary experiment using macroarray technology (Atlas Human Cancer 1.2 macroarray, Clontech, Palo Alto, CA, USA) to gain access to a rapid overview of genes differentially expressed in the resistant phenotype. Comparing the gene expression between the most resistant clone HCT116/R2 and the sensitive clone HCT116/S, this macroarray analysis showed alterations in the apoptotic pathway. In particular, the most dramatic change was a marked down-regulation of the Bax gene in resistant HCT116/R2 cells (data not shown). On the basis of the results of this preliminary exploration, we decided to focus our attention on the apoptotic pathway to examine whether oxaliplatin resistance could be associated with dysregulation of apoptosis.

To this end, we evaluated by FACS analysis the ability of the three clones HCT116/S, HCT116/R1 and HCT116/R2 to undergo apoptosis in response to oxaliplatin treatment. To observe pharmacologically relevant effects, we tested oxaliplatin at a concentration of 15 μM, which corresponds approximately to the plasma peak in patients and leads in vitro to full activation of apoptosis in sensitive cells. As shown in Fig. 1, oxaliplatin induced apoptosis in the sensitive HCT116/S cells, but it failed to induce apoptosis in the resistant HCT116/R1 and HCT116/R2 clones. Similar results were observed at 48, 72 and 96 h after the beginning of oxaliplatin treatment (data not shown at 72 and 96 h).

We questioned whether or not resistance to apoptosis was specific for oxaliplatin. For this purpose, cells were treated with 35 μM irinotecan, which is the concentration we found to be equitoxic to 15 μM oxaliplatin in HCT116/S cells. Fig. 1 shows that the resistant cells were able to undergo apoptosis when treated with irinotecan but to a lesser extent than the HCT116/S cells. The differential sensitivity to irinotecan-induced apoptosis in HCT116/R1 and HCT116/R2 cells observed in Fig. 1 is consistent with the low cross-resistance between oxaliplatin and irinotecan of the HCT116/R1 cells (Table 1). These data show that the resistant phenotypes are specifically associated with a decrease in oxaliplatin-induced apoptosis. The question remains as to whether this resistance results from a lower level of injury in the resistant cells or whether it is due to an alteration of apoptosis. To address this question, we tested the cellular sensitivity to the apoptosis inducers lonidamine and arsenic trioxide, known to act directly on the mitochondrial pathway of apoptosis by opening the permeability transition pore [17,18].

3.3. *Concomitant resistance to lonidamine- and arsenic trioxide-induced apoptosis in oxaliplatin-resistant phenotypes*

To investigate a possible defect in the mitochondrial apoptotic pathway, we examined the ability of lonidamine and arsenic trioxide to induce apoptosis in the resistant cells (HCT116/R1 and HCT116/R2). The percentage of apoptotic cells was evaluated after treatment with 220 μM lonidamine or 40 μM arsenic trioxide for 24 h (optimal conditions for inducing apoptosis in the sensitive clone). Fig. 2A shows that lonidamine induced marked apoptosis in the sensitive cells HCT116/S, whereas it only induced limited apoptosis in HCT116/R1 and no detectable apoptosis in HCT116/R2 cells. Similarly, Fig. 2B shows that arsenic trioxide induced marked apoptosis in the sensitive cells, whereas it only induced limited apoptosis in HCT116/R1 and slightly detectable apoptosis in HCT116/R2. This last experiment, by directly measuring the depolarization of the mitochondrial membrane, demonstrated that there is indeed a functional alteration in the mitochondrial apoptotic pathway not only in the R2 clone but also in the R1 resistant cell line. The correlation between resistance to oxaliplatin-induced apoptosis and resistance to lonidamine- or

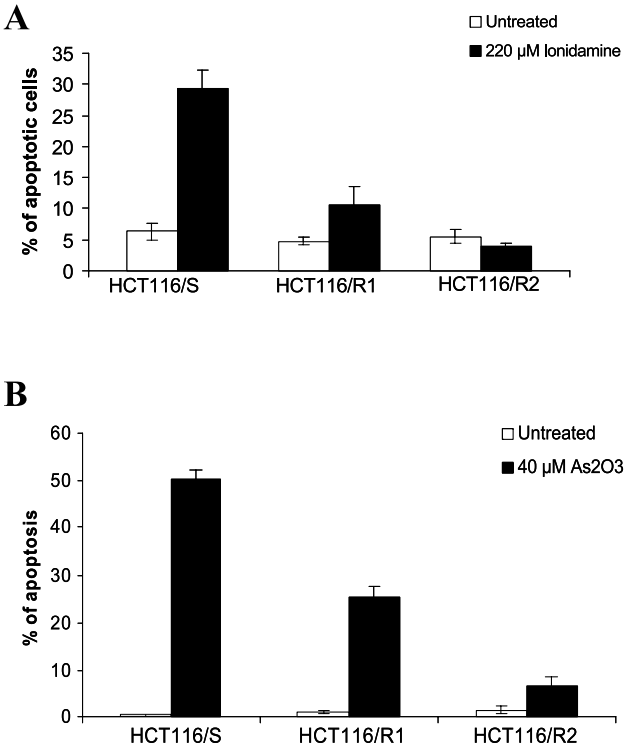

Fig. 2. Susceptibility to lonidamine- or arsenic trioxide-induced apoptosis in resistant clones. Cells were treated for 24 h with 220 μM lonidamine (A) or 40 μM arsenic trioxide (As_2O_3) (B). The percentage of cells entering apoptosis was determined by FACS analysis using FITC-labeled annexin V (A) or the MitoCapture apoptosis detection kit (BioVision), which detects the changes in the mitochondrial transmembrane potential (B). Results are the means of three independent experiments; bars, S.D.

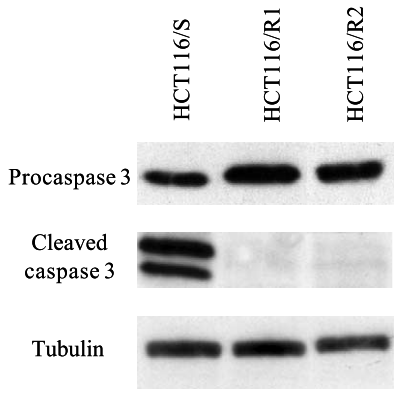

Fig. 3. Western blotting of activated caspase 3 in HCT116 variants after oxaliplatin treatment. Cells were treated with 50 μM oxaliplatin for 24 h before lysate preparation for immunoblot analysis as described in Section 2. Expression of tubulin was used to control equivalent protein loading and transfer.

arsenic trioxide-induced apoptosis in the resistant cells strongly argues in favor of a defect in the mitochondrial apoptotic pathway which could contribute to resistance to oxaliplatin.

3.4. Molecular analysis of caspase 3, Bax, Bak, Bcl-2 and Bcl-X_L

To further assess the contribution of a defect in the mitochondrial apoptotic pathway to the resistance to oxaliplatin, we studied five key apoptotic effectors, namely, caspase 3, Bax, Bak, Bcl-2 and Bcl-X_L [19,20].

In the absence of treatment, caspase 3 processing was not detectable in either of the HCT116 variants (data not shown). Patterns of caspase 3 activation in the HCT116 variants following oxaliplatin treatment are shown in Fig. 3. Following treatment with 50 μM oxaliplatin, activated caspase 3 was detected as two fragments (approximately M_r 19 000 and M_r 17 000) in sensitive HCT116/S cells. In contrast, caspase 3 activation was undetectable in the two resistant clones HCT116/R1 and HCT116/R2. This difference is not due to a defect in caspase 3 expression since the procaspase expression levels were found to be identical in the three cell lines (Fig. 3). These results demonstrate an association between resistant phenotypes and loss of caspase 3 activation, which is consistent with the resistance to oxaliplatin-induced apoptosis demonstrated by FACS analysis.

We also investigated Bax expression, given the specificity of Bax for induction of the mitochondrial apoptotic pathway. Fig. 4A shows that Bax expression was detectable and markedly induced following oxaliplatin treatment in HCT116/S and HCT116/R1 cells. In the HCT116/R1 variant, resistance thus did not seem to be due to a modulation of Bax expression. In contrast, in the most resistant HCT116/R2 clone, Bax was undetectable independently of oxaliplatin treatment. Loss of Bax expression confirmed and extended the mitochondrial apoptosis defect associated with oxaliplatin resistance in HCT116/R2 cells. As a high Bcl-2/Bax ratio is known to favor cell survival, whereas a low ratio promotes apoptosis, we investigated Bcl-2 expression (Fig. 4A). Unlike Bax, Bcl-2 expression remained unchanged in sensitive, resistant and cells both in the absence and in the presence of oxaliplatin. We also investigated expression of the pro-apoptotic Bak and the anti-apoptotic Bcl-X_L in order to further characterize the mitochondrial apoptotic response (Fig. 4B). In the absence of oxaliplatin treatment, Bak and Bcl-X_L expression was similar in the three cell lines. The two proteins were induced by oxaliplatin treatment but at the same level in the three cell lines. These findings suggest that the modulation of Bak, Bcl-2 or Bcl-X_L expression is not involved in the acquisition of oxaliplatin resistance in our model.

3.5. Sequencing of the Bax gene

Loss of Bax expression was observed in the most resistant phenotype. An inherent instability in a G8 tract of the Bax gene has been demonstrated to be a mutational target in the case of MMR-deficient colorectal cancers [15]. Moreover, mutations in this G8 tract of the Bax gene are particularly frequent in the HCT116 cell line. In addition, such a mutation was recently suggested to play a key role in the acquisition of a profound resistance to non- steroid anti-inflammatory drugs that could help prevent colon polyps from becoming cancerous [21]. To determine whether the loss of Bax expression in HCT116/R2 cells was related to a frameshift mutation in the Bax G8 tract spanning codons 38–41, we sequenced this region of the Bax gene in all the HCT116 variants. Sequence analysis revealed that both Bax alleles were mutated in the HCT116/R2 clone resulting in a deletion of a G base in the G8 tract (G7/G7 homozygous), whereas the HCT116/S and HCT116/R1 clones were heterozygous and conserved one wild-type Bax allele (G8/G7).

4. Discussion

Molecular mechanisms involved in oxaliplatin resistance remain to be characterized. In this study we established a cel-

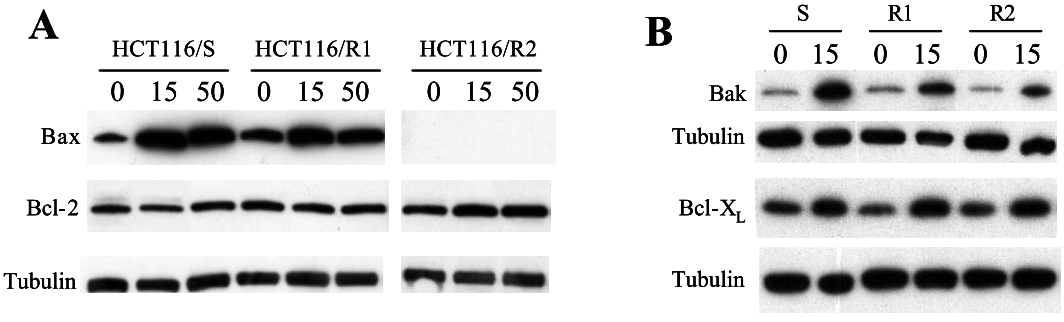

Fig. 4. Western blotting of Bax, Bak, Bcl-2 and Bcl-X_L in HCT116 variants prior to or following oxaliplatin treatment. Cells were untreated or treated with 15 μM oxaliplatin for 48 h or with 50 μM oxaliplatin for 24 h before lysate preparation. Immunoblot analyses were performed as described in Section 2. Expression of tubulin was used to control equivalent protein loading and transfer

lular model based on the oxaliplatin-sensitive human colon carcinoma HCT116 cell line. By growing the cells in increasing concentrations of oxaliplatin, we were able to isolate cell lines 30- and 70-fold more resistant than the sensitive clone HCT116/S. The specificity of this cellular model for oxaliplatin was demonstrated by limited cross-resistance to two commonly used anticancer drugs.

By FACS analysis we demonstrated that susceptibility to apoptosis induced by oxaliplatin was specifically decreased in resistant cells. Alteration in the activation of caspase 3 following oxaliplatin treatment confirmed the reduced apoptosis in the resistant cells. The observed concomitant resistance to lonidamine-induced apoptosis suggests a defect within the mitochondrial apoptotic pathway in the resistant cells. These data are strongly supported by the cross-resistance to apoptosis induced by arsenic trioxide, another agent able to induce apoptosis via the mitochondria [18].

Loss of Bax expression was observed only in the most resistant phenotype suggesting that Bax probably represents only one of the putative mitochondrial altered factors, since it does not seem to be involved in the resistance of the HCT116/R1 cells. Nevertheless, these cells exhibited partial cross-resistance to lonidamine- and arsenic trioxide-induced apoptosis, suggesting an alteration in the mitochondrial apoptotic pathway.

To date, the molecular mechanisms underlying resistance to oxaliplatin have only been studied in a cellular model derived from the A2780 ovarian carcinoma cell line. Two mechanisms have been associated with oxaliplatin resistance in this cellular model: an increase in the γ-glutamyl transpeptidase activity leading to elevation in cellular GSH [12] and a reduction in platinum accumulation and DNA–platinum adduct levels [13]. Nevertheless, the contribution of apoptosis to the resistance to oxaliplatin has never been described before and represents an interesting new pathway to investigate further. Indeed, anticancer drugs have typically been thought to induce apoptosis by affecting intrinsic death pathways that converge to the release of apoptogenic molecules from the mitochondria into the cytosol [22,23]. The ability of cells to maintain a balance in favor of apoptotic cell death appears to be an important determinant of efficacy in many anticancer chemotherapies, and alterations in apoptotic pathways may often be associated with treatment failure. Strategies to overcome drug resistance by modulating apoptosis are now under investigation with several clinical trials in progress [24].

In conclusion, our study shows that acquisition of resistance to oxaliplatin was associated with alterations within the mitochondrial apoptotic pathway in our cellular model. This report underlines the fact that perturbations within the mitochondrial apoptotic pathway may contribute to oxaliplatin resistance and that Bax could contribute to this apoptotic defect in some cases. Further studies are needed to clarify the involvement of mitochondrial apoptosis in oxaliplatin resistance in this and other colorectal cancer cell models.

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