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SHORT REPORTS

Low daunomycin concentrations protect colorectal cancer cells from hypoxia-induced apoptosis

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Hypoxia, a common feature of solid tumors, is a direct stress that triggers apoptosis in many cell types. Poor or irregular tumor vascularization also leads to a decreased drug diffusion and cancer cells distant from blood vessels (hypoxic cells) are exposed to low drug concentrations. In this report, we show that low daunomycin concentrations protect HCT116 colorectal cancer cells from hypoxia-induced apoptosis. While hypoxia induced p53 accumulation without expression of its responsive genes (*bax* and *p21*), daunomycin treatment restored *p53* transactivation activity and cell cycle progression. We also demonstrated a role for Akt activation in daunomycin-induced protection through phosphorylation and inactivation of the Bcl-2 family proapoptotic factor Bad. Our data therefore suggest that chemotherapy could possibly, because of low concentrations in poorly vascularized tumors, protect cancer cells from hypoxia-induced cytotoxicity.
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Hypoxic solid tumors have a poorer prognosis than well-oxygenated tumors, independently of treatment. There is experimental evidence that the tumor microenvironment affects both the malignant progression of transformed cells and their responsiveness to chemo- and radiotherapy (Brown and Giaccia, 1998; Vaupel *et al*., 2001). Hypoxia may influence tumor biology in opposing ways: it is toxic as a stress; but on the other hand, hypoxic solid tumors contain viable cells, which are particularly resistant to treatment and contribute to disease relapse. Indeed, hypoxia is rapidly toxic in most cell types as it can induce necrosis or apoptosis in normal or in transformed cells (Yamaguchi *et al*., 2001; Zhu *et al*., 2002). Cell death is particularly prominent in the zones farthest from the tumor vasculature, where hypoxia stimulates apoptosis (Graeber *et al*., 1996). Several studies showed that poor O_2 supply resulted in *p53*-dependent programmed cell death (Graeber *et al*., 1994). However, hypoxia may also be involved in the development of a more aggressive phenotype and contribute to metastasis. In experimental tumors, hypoxia can provide a selective pressure for the expansion of oncogenically transformed cell populations with a reduced apoptotic response to hypoxia as well as to chemotherapeutic agents (Graeber *et al*., 1996; Kim *et al*., 1997). The protective effect of hypoxia against radio- or chemotherapy-induced cytotoxicity has been extensively explored (Ogiso *et al*., 2000; Vaupel *et al*., 2001; Achison and Hupp, 2003).

Moreover, poor vascularization impairs chemotherapeutic compound delivery by constricted blood vessels and hypoxic cancer cells distant from blood vessels are exposed to low drug concentration. Therefore, we investigated the effect of low daunomycin concentrations on hypoxia-induced apoptosis in HCT116 colorectal adenocarcinoma cells.

HCT116 human colorectal adenocarcinoma cells were tested for viability in severe hypoxia. Hypoxic cells were grown in a conditioned atmosphere where O_2 was maintained below 0.1%. Cell viability experiment revealed that only 14.2% of HCT116 hypoxic cells were still alive after 72 h as compared to normoxic controls (Figure 1a).

Surprisingly, cotreatment of these cells with very low daunomycin concentrations protected them against hypoxia-induced toxicity. Indeed, 81.2% of cells cotreated with 0.01 μM of daunomycin were still alive after 3 days of hypoxia ($P = 0.0005$). Similarly, following 48 h of daunomycin and hypoxia, cells replated in normoxia could grow again while cells exposed to hypoxia alone died (data not shown). This 'protective effect' is concentration dependent and was less pronounced with higher daunomycin concentrations (68% of hypoxic cell viability with 0.05 μM daunomycin; and 44.5% with 0.1 μM daunomycin). Indeed, daunomycin intrinsic cytotoxicity at these higher concentrations reduced HCT116 cell viability in normoxic conditions (Figure 1a).

As hypoxia is a well-known inducer of apoptosis, cell extracts from hypoxic cells were analysed for PARP and caspase-3 cleavage and activation (Figure 1b; top and

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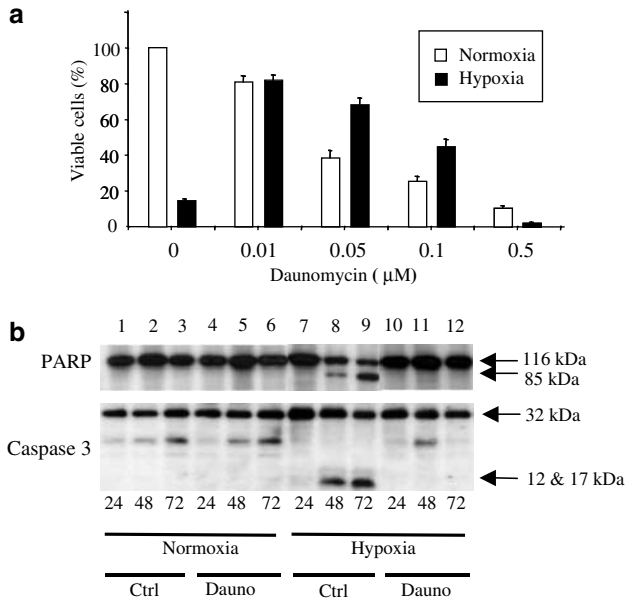

Figure 1 Low daunomycin concentrations protect HCT116 adenocarcinoma cells from hypoxia-induced apoptosis. (**a**) HCT116 cells (ATCC CCL 247) were treated with increasing daunomycin concentrations (0.01–0.5 μM) and incubated in hypoxia (black columns) or normoxia (white columns) for 3 days. Cell viability was assessed with the WST-1 test (Boehringer Mannheim, Mannheim, Germany). Each value represents the mean of three independent experiments as compared to normoxic controls (100%). Hypoxia was achieved with the PROOX 110 oxygen controller, which provides oxygen control from 0.1 to 99.9% inside one insert culture chamber sitting in a CO_2- and temperature-controlled incubator. Tissue culture dishes were placed in the semisealed culture chamber and severe hypoxia (<0.1% O_2) was achieved by injection of a N_2 95%–CO_2 5% mixture. The oxygen tension in the chamber was measured by an O_2 sensor and maintained below 0.1% O_2 by PROOX 110. (**b**) Daunomycin inhibits hypoxia-induced PARP and caspase-3 cleavage in HCT116 cells. Cells were left untreated (Ctrl) or were treated with 0.01 μM of daunomycin (Dauno, Rhône Poulenc Rorer-Brussels) and incubated in hypoxia or normoxia for the indicated times. Total cell lysates were made by resuspending the cellular pellets in lysis buffer (HEPES 25 mM, NaCl 150 mM, Triton 0.5%, Glycerol 10%, DTT 1 mM, sodium orthovanadate 1 mM, β-glycerophosphate 25 mM, sodium fluorure 1 mM, and protease inhibitors) and were analysed by Western blotting with antibodies recognizing PARP (mouse anti-poly(ADP-ribose)polymerase monoclonal antibody, Oncogene Research Products) or caspase-3 (mouse anti-caspase-3 monoclonal antibody, Alexis Biochemichals). Arrows indicate the full-length (116 kDa) and cleaved (85 kDa) PARP as well as the full-length (32 kDa) and activated (17 and 12 kDa) caspase-3

bottom panels, respectively). Caspase-3 activation and PARP cleavage were observed after 48 or 72 h of hypoxia (Figure 1b, top and bottom panels, lanes 8 and 9). However, hypoxic HCT116 cells did not undergo apoptosis in the presence of daunomycin, (0.01 μM) as in these conditions, we could not observe any caspase-3 activation or PARP cleavage (Figure 1b; top and bottom panels, lanes 11 and 12).

Several groups have previously demonstrated that multiple pathways can stabilize p53 in response to different forms of stress (Ashcroft *et al*., 2000). p53 activation by DNA damage is followed by increased synthesis of downstream effector proteins. Hypoxic stress, like DNA damage, induces p53 protein accumulation and p53-dependent apoptosis in transformed cells (Koumenis *et al*., 2001), but several reports indicated that hypoxia-induced p53 has lost its transactivation potential and rather behaves as a transcriptional repressor in tumor cells. To address p53 expression and activity in our experimental model, nuclear and total protein extracts were prepared after 24, 48, and 72 h of hypoxia or hypoxia plus daunomycin treatment, and expressions of p53 and of two p53-responsive proteins, p21 and Bax, were investigated by Western blot analysis.

Although hypoxia induced a strong p53 accumulation in HCT116 cells (Figure 2, top panel, compare lane 1 with lanes 5–7), we did not observe any p21 expression in these conditions (Figure 2, second panel, compare lane 1 with lanes 5–7). However, daunomycin treatment (0.01 μM) of hypoxic HCT116 cells resulted in a shorter and reduced p53 accumulation, suggesting a link between the protective effect and p53 destabilization (Figure 2, top panel, lanes 8–10).

Interestingly, we observed a slight increase of p21 expression in hypoxic cells cotreated with 0.01 μM of daunomycin, thus indicating restoration of a p53 transcriptional activity (Figure 2, second panel, lanes 8–10). A similar observation was obtained with higher daunomycin concentrations (0.05 μM) (data not shown).

Hypoxia also failed to induce the accumulation of the Bax protein while daunomycin treatment did (Figure 2, third panel from the top). Cotreatment of hypoxic cells with daunomycin 0.01 μM (or 0.05 μM; data not shown) resulted in Bax accumulation (Figure 2, third panel from the top, lanes 8–9). These results thus suggest that hypoxia induces the accumulation of a transcriptionally latent p53 that becomes transcriptionally active upon daunomycin treatment.

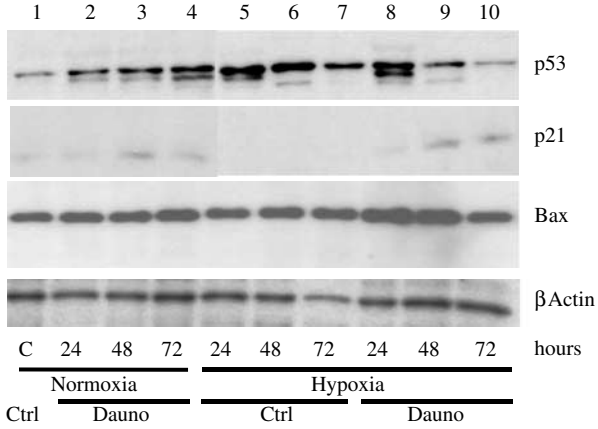

Figure 2 p53, p21, and Bax expression in response to hypoxia and daunomycin. HCT116 cells were left untreated (Ctrl) or were treated with 0.01 μM of daunomycin (Dauno) and incubated in hypoxia or normoxia for the indicated times. Total cell lysates were analysed by Western blotting for p53 (mouse anti-p53 monoclonal antibody Ab2, Oncogene Science), p21 (mouse anti p21/WAF 1 Ab-1 monoclonal antibody, Oncogene Research Products), or Bax (mouse anti-Bax monoclonal antibody, Oncogene Research Products) expression

Since transition between apoptotic cell death and proliferation in stress conditions is regulated at cell cycle checkpoints, we determined whether enhanced p21 expression induced a cell cycle arrest that prevented hypoxia-induced apoptosis. The cell cycle parameters were thus monitored under hypoxic conditions in the presence or absence of low daunomycin concentrations. Hypoxia led to an increased number of cells in S phase and ultimately to apoptosis (Figure 3a, third panel). Simultaneous daunomycin treatment (0.01 μM) restored cell cycle progression after a transient accumulation in S phase (Figure 3a, fourth panel). These data indicate that daunomycin releases cells from hypoxia-induced S-phase arrest.

To investigate whether p21 expression was required for the daunomycin-mediated protective effect, p21-deficient HCT116 cells (HCT116 p21−/−) were tested for viability after 3 days of hypoxia in the presence or absence of daunomycin (Figure 3b and c). HCT116 p21−/− cells were sensitive to hypoxia-induced apoptosis and were protected, similarly to the wild-type cells, by treatment with 0.01 μM of daunomycin (Figure 3c; $P = 0.003$). p21 is effectively not expressed in HCT116 p21−/− cells as demonstrated by Western blotting (Figure 3b, top panel lanes 1–10). Therefore, p21 did not play any major role in the daunomycin-induced protective effect.

Hypoxia influences a variety of signal transduction pathways, including MAP kinases (Erk, JNK, and p38) and PI3K/Akt kinases. Akt is a serine–threonine kinase involved in proliferative, metabolic, and apoptotic pathways and is critical for cell survival (Datta *et al*., 1999; Nicholson and Anderson, 2002). Activated phospho-Akt (P-Akt) has been shown to protect normal and tumoral cells against hypoxia and p53-induced apoptosis (Ogawara *et al*., 2002). Our data showed that

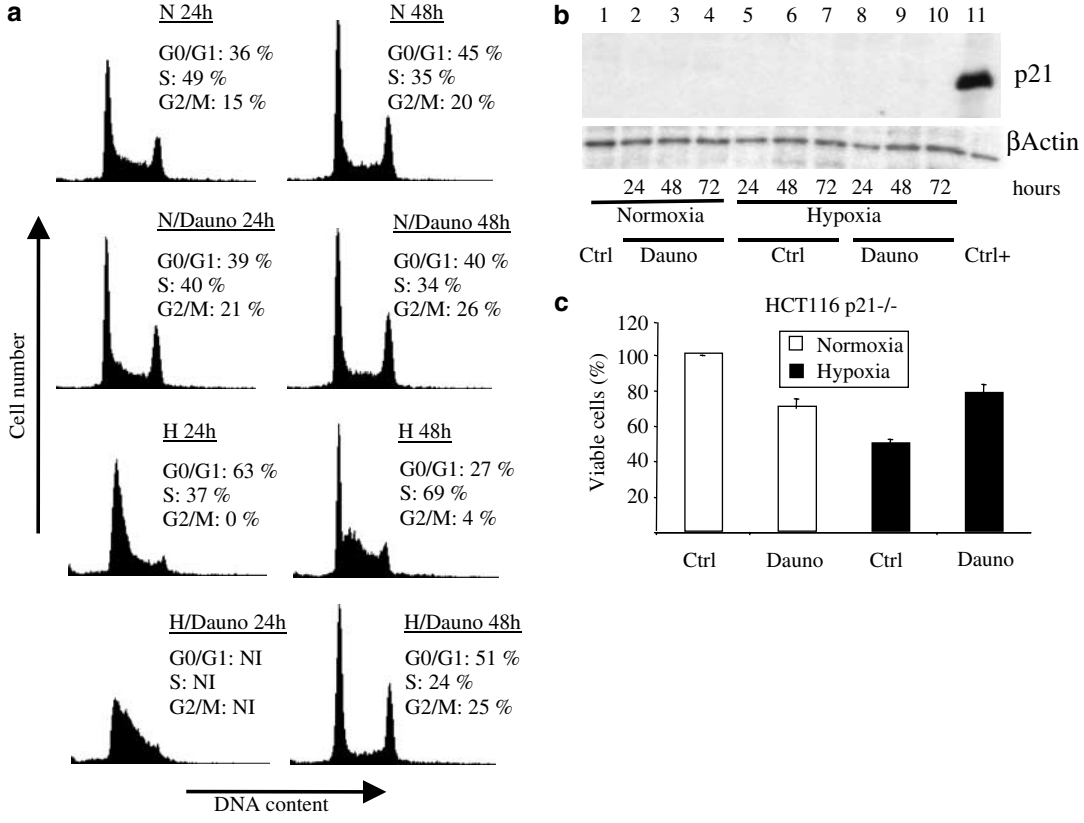

Figure 3 Cell cycle distribution and p21 role in HCT116 cells hypoxia-induced apoptosis and daunomycin protection. (**a**) Cell cycle distribution. HCT116 cells were left untreated or were treated with 0.01 μM of daunomycin (Dauno) and incubated in hypoxia (H) or normoxia (N) for the indicated times. Cells were then stained with propidium iodide in the presence of RNAse A as recommended by the manufacturer (Cycle Test Plus DNA reagent kit ; Becton Dickinson, European HQ, Erembodegem, Belgium). Cells were analysed with a FACStar plus flow sorter (Becton) with a 100-mW air-cooled argon laser (spinnaker 1161; Spectra Physics, Mountain View, CA, USA) and the CellQuest software (Macintosh, Facstation; Becton Dickinson). DNA histograms were analysed by MODFIT LT flow cytometry modeling software (Verity software house, Topsham, ME, USA). A representative result out of three independent experiments is shown (NI: noninterpretable). (**b**) HCT116 p21−/− cells do not express p21. HCT116 p21−/− cells, kindly provided by Drs Yu and Vogelstein (John Hopkins University), were left untreated (Ctrl) or treated with 0.01 μM of daunomycin (Dauno) and incubated in hypoxia or normoxia for the indicated times (Ctrl+: wild-type HCT116 + daunomycin). Total cell lysates were analysed by Western blotting for the expression of p21. (**c**) HCT116 p21−/− cells in hypoxia. HCT116 p21−/− cells were left untreated (Ctrl) or treated with 0.01 μM daunomycin (Dauno) and incubated in hypoxia (black columns) or normoxia (white columns) for 3 days. Cell viability was assessed with the WST-1 test. Each value represents the mean of three independent experiments as compared to untreated normoxic cells (100%)

hypoxia did not induce Akt activity in HCT116 cells (Figure 4a, top panel from the top, lanes 5–7). However, when hypoxic cells were treated with low daunomycin concentrations, we observed a progressive increase of P-Akt level (Figure 4a, top panel, lanes 8–10), whereas total Akt expression remained unchanged (Figure 4a, bottom panel). Moreover, LY294002, an Akt specific inhibitor, abolished both the Akt phosphorylation and the daunomycin protective effect (Figure 4a first panel, lanes 11–13 and 14–16 and Figure 4c, bottom panel) in a cell viability assay.

An important if not dominant mechanism for phospho-Akt-induced protection comes from its ability to block the activation of Bad-dependent cell death pathway. Akt-induced Bad phosphorylation inactivates Bad ability to form heterodimers with antiapoptotic Bcl-2 proteins, a step thought to be prominent in Bad proapoptotic effects (Datta *et al*., 1997; del Peso *et al*., 1997).

Phospho-Bad analysis by Western blotting correlated Akt and Bad phosphorylation. Indeed, while hypoxia did not induce Bad phosphorylation in HCT116 cells, we observed a progressive Bad phosphorylation when hypoxic cells were treated with low daunomycin concentration (Figure 4b, top panel, lanes 5–7). Moreover, cell treatment with LY 294002 abolished Bad phosphorylation (Figure 4b, top panel, lanes 8–10 and 11–13), as previously described (Datta *et al*., 1997), thus confirming that Bad phosphorylation was Akt dependent in our model.

Although hypoxia is toxic for most cell types, numerous studies report that hypoxia induces tumor cell drug resistance through various mechanisms (Sanna and Rofstad, 1994; Teicher, 1994; Tomida and Tsuruo, 1999). Poor vascularization impairs chemotherapeutic compound delivery and cancer cells distant from blood vessels (hypoxic cells) are exposed to lower drug concentration. However, little was known about the effect of low drug concentrations on hypoxia-induced apoptosis. In this study, we report that low concentrations of daunomycin, a common anticancer agent, inhibit hypoxia-induced apoptosis in HCT116 colorectal adenocarcinoma cells. Hypoxia triggered p53 accumulation without induction of its responsive genes, while daunomycin-induced protection correlated with reduced p53 and restored p21 expression.

Controversial results have been reported on p53 involvement in hypoxia-induced apoptosis. Achison and Hupp (2003) paradoxically showed that p53 loss sensitized HCT116 cells to hypoxia-induced cell death and that hypoxia attenuated p53 activation by 5-fluorouracile. Conversely, in our study, p53-accelerated degradation seems to be linked to daunomycin cytoprotection. Experimental differences may account for these discording results. In Achison's study, cells were exposed to 1% of O_2, while we lowered the oxygen concentration to $< 0.1\%$ O_2 reaching severe hypoxia. Our results are in agreement with other studies. Indeed, HCT116 p53−/− cells have lost their ability to undergo apoptosis under hypoxia (Yu *et al*., 2002), while only severe hypoxia induces p53-dependent apoptosis (Koumenis *et al*., 2001).

All these studies agree that hypoxia-induced p53 is not transcriptionally active. Indeed, Koumenis *et al*.

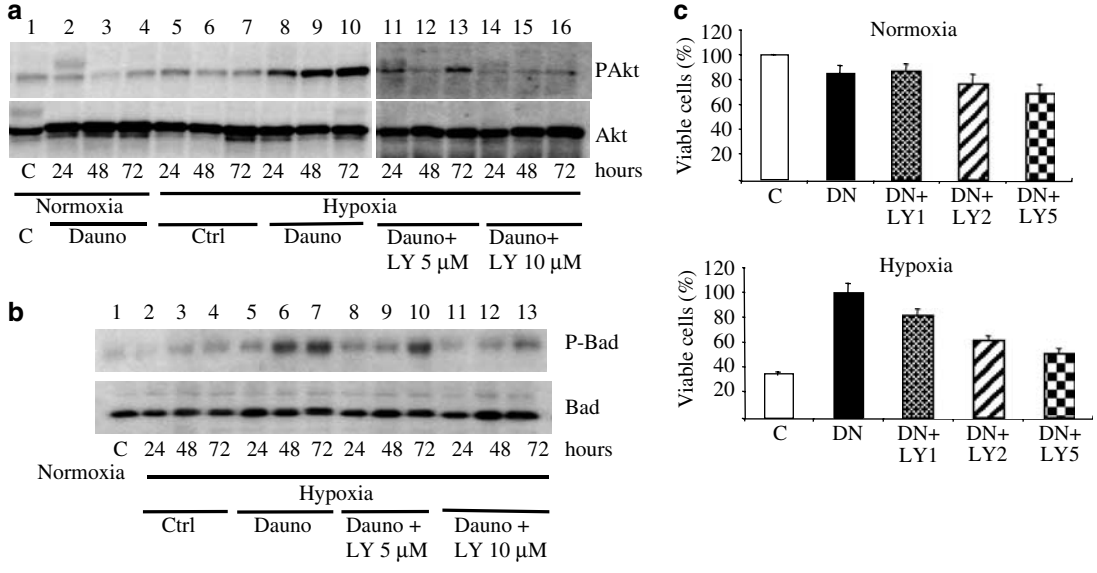

Figure 4 Akt-dependent Bad phosphorylation and daunomycin-induced protection against hypoxia-induced apoptosis. (**a**, **b**) HCT116 cells were left untreated (Ctrl) or were treated with 0.01 μM of daunomycin (Dauno) and incubated in hypoxia or normoxia for the indicated times, in the absence or presence of LY294002 (LY, Alexis Biochemicals, dissolved in DMSO). Total cell lysates were analysed by Western blotting for the expression of Akt, phospho-Akt (**a**) (rabbit anti-Akt and anti-phospho-Akt (Ser 473) polyclonal antibodies, Cell Signaling), Bad and phospho-Bad (**b**) (rabbit anti-Bad and anti-phospho-Bad polyclonal antibodies, Cell Signalling). (**c**) HCT116 cells were treated with daunomycin (DN) and incubated in hypoxia or normoxia for 3 days. This treatment was performed in the absence or presence of LY294002 (LY; 1, 2, or 5 μM). Cell viability was assessed with the WST-1 test. Each value represents the mean of three independent experiments as compared to normoxic controls (100%)

demonstrated that, in contrast to DNA damage, hypoxia fails to induce endogenous downstream p53-regulated mRNAs and proteins despite p53 accumulation and our results showed that the expression of the p53-responsive genes *p21* and *bax* is not induced in HCT116 hypoxic cells.

Despite a lower p53 expression, daunomycin treatment restored *bax* and *p21* induction under hypoxia, suggesting a restoration of p53 transcriptional activity in hypoxic cells as previously described with doxorubicin, another topoisomerase II inhibitor (Koumenis *et al*., 2001). However, in the referred report, doxorubicine induced apoptosis by itself and the addition of doxorubicine prior to hypoxic treatment generated a substantial increase in the apoptotic rates. Indeed, these authors used cytotoxic doxorubicine concentrations, while we worked with low nontoxic concentrations.

Recent papers have focused on an antiapoptotic role of p21 in several cellular models indicating that the choice between growth arrest and apoptosis in HCT116 colorectal cancer cells can be modulated by p21 (Yu *et al*., 2003). However, in our study, p21 did not have a major role in the daunomycin protective effect as p21 disruption in HCT116 cells (HCT116 *p21*−/−) did not modify cell response to hypoxia and daunomycin.

PI3K/Akt pathway seems implicated in the protective effect observed in our experimental conditions. Beneficial effects of Akt activation against hypoxic or ischemic insults have been described in cardiomyocytes and astrocytes (Ruscher *et al*., 2002; Chao *et al*., 2003). Although there can be little doubt that PKB/Akt promotes cell survival, the mechanisms involved have only recently begun to emerge. In addition to influencing the transcription of pro- and antiapoptotic genes, numerous studies indicate that PKB/Akt promotes survival by directly phosphorylating key regulators of the apoptotic cascade (Datta *et al*., 1999; Nicholson and Anderson, 2002). Bad, a member of the Bcl-2 family, which promotes apoptosis by binding to and antagonizing the action of the prosurvival members of the family such as Bcl-2 or Bcl-Xl, can be phosphorylated and inhibited by Akt. Indeed, S136 phosphorylation promotes the sequestration of Bad by 14-3-3 proteins in the cytosol, thus preventing Bad from interacting with Bcl-2 or Bcl-Xl (Datta *et al*., 1997; del Peso *et al*., 1997). The biological importance of Bad phosphorylation on Ser 136 in promoting survival has been demonstrated after several stress signals. For example, Hirai *et al*. (2004) demonstrated that Bad phosphorylation by Akt in brain slices prevented cytochrome *c*-associated apoptosis after hypoxia. NF-κB could also be induced by Akt activation. However, we do not have any evidence that NF-κB plays a role in Akt-dependent cytoprotective effect as this activity is preserved in HCT116 cells stably expressing an NF-κB super-repressor (data not shown).

Our data indicate that low daunomycin concentrations induced Bax expression in hypoxic cells but inhibited apoptosis. This observation is indeed surprising and different hypotheses could possibly explain it. As mentioned, Akt-dependent Bad phosphorylation promotes its sequestration in the cytosol. The induction of apoptosis requires a positive ratio between proapoptotic and antiapoptotic members of the Bcl-2 family. Possibly, Bax increased expression is not sufficient to induce apoptosis in the absence of mitochondrial Bad. Alternatively, Akt could induce the expression of apoptosis inhibitors acting downstream of Bax. Indeed, data suggesting that Akt may influence post-mitochondrial events have been reported as Akt was shown to inhibit the activation of caspase-3 and -9 by released cytochrome *c* through unidentified cytosolic factors (Nicholson and Anderson, 2002). Finally, it is not clear whether Bax is really involved in hypoxia-induced apoptosis and Bax induction is not sufficient for p53-induced apoptosis (Sakamuro *et al*., 1997).

To our knowledge, this is the first study reporting that daunomycin prevents hypoxia-induced apoptosis through activation of survival pathways, thus indicating a novel mechanism explaining that cancer cells can resist to a natural cytotoxic stress. Moreover, some reports indicate that other drugs such as traztuzumab, tamoxifen, or doxorubicin could also activate the PI3K/Akt pathway leading to resistance to these drugs (Plo *et al*., 1999; Clark *et al*., 2002) and possibly also, as suggested by our data, to hypoxia-induced apoptosis. The ability to tolerate hypoxia and withstand apoptosis may be required for tumor progression. PKB/Akt, with its critical role in cell survival, may have an important function in adaptation to these adverse environmental changes.

While it has been widely reported that cancer cell hypoxia can lead to tumor resistance to chemotherapy and radiotherapy, our data indicate that in some cancer cells, the administration of a chemotherapy could favor the resistance of poorly vacularized tumors to hypoxia. Therefore, it is quite possible that chemotherapy could promote the survival of hypoxia-exposed cancer cells and thus select such cells, thereby facilitating subsequent cancer relapse. If such data could be confirmed in other models, it would strongly support the administration of oxygenation-boosting agents simultaneously with chemotherapy. Alternatively, inhibition of the PI3K/Akt pathway could prevent the cytoprotective effect of low local drug concentrations as suggested by a gene therapy study demonstrating that *in vivo* expression of PTEN in a model of the human bladder suppresses tumor growth, downregulates phosphorylated Akt, and increases sensitivity to doxorubicin (Tanaka and Grossman, 2003).

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