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Metastatic SW620 colon cancer cells are primed for death when detached and can be sensitized to anoikis by the BH3-mimetic ABT-737

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Anoikis, a Bax-dependent apoptosis triggered by detachment from the extracellular matrix, is often inhibited in metastatic cancer cells. Using a couple of isogenic human colon cancer cell lines derived either from the primary tumor (SW480) or from a lymph node metastasis (SW620), we found that only SW480 cells were sensitive to anoikis. Bim upregulation but not Mcl-1 degradation was determined to be a critical factor of anoikis initiation in SW480 cells. ERK-mediated phosphorylation targets Bim for ubiquitination and proteasomal degradation. A MEK inhibitor (PD0325901) was able to increase Bim expression in SW620 cells and to sensitize these cells to anoikis. Thus, in both cell lines anoikis is under the control of proteins of the Bcl-2 family. Most interestingly, the BH3-mimetic ABT-737 was found not only to increase the level of apoptosis in suspended SW480 cells but also to sensitize SW620 cells to anoikis. Accordingly, both cell lines cultured in suspension were found to be primed for death, as determined by the detection of Bcl-2:Bim and Bcl-xL:Bim complexes. In contrast, adherent SW480 and SW620 cells were resistant to ABT-737. This indicates that, whether or not they undergo anoikis, colon cancer cells that have detached from the extracellular matrix might go through a transient state, where they are sensitive to BH3 mimetics. This would confer to compounds such as Navitoclax or ABT-199 a therapeutic window where they could have anti-metastatic potential.

Anoikis is a particular apoptotic death due to loss of appropriate cell adhesion,[1–3] and tumor cells that acquire metastatic potential have developed mechanisms to resist anoikis.[4,5] In spite of its unique definition, anoikis is essentially an apoptotic process. In keeping with classical apoptosis, anoikis involves either the intrinsic pathway, due to the perturbation of mitochondrial homeostasis, or the extrinsic pathway triggered by cell surface death receptors.[3,6]

Proteins of the B-cell leukemia/lymphoma 2 (Bcl-2) family are key arbiters of the commitment to apoptosis at the mitochondrion.[2,7] This family consist of both pro- and anti-apoptotic members, which all share sequence homology in their Bcl-2 homology (BH) domains. The anti-apoptotic proteins, including Bcl-2, Bcl-2-related gene, long isoform (Bcl-xL), Bcl-w and myeloid cell leukemia 1 (Mcl-1) contain BH domains 1–4. They are generally found on the outer mitochondrial membrane, where they function to inhibit the pro-apoptotic Bcl-2 proteins. The pro-apoptotic proteins are divided into the multi-domain effectors Bcl-2-acssociated x protein (Bax), Bak and Bok (containing BH domains 1–3), or BH3-only proteins, which contain only the BH3 domain. The multi-domain pro-apoptotic proteins Bax and Bak promote mitochondrial outer membrane permeabilization.[8]

The BH3-only proteins, such as Bcl-2 interacting domain death agonist (Bid), Bim and PUMA act as either direct activators of pro-apoptotic Bax and Bax, or as repressors of anti-apoptotic proteins.[9,10] They have been named activators. The other class of BH3-only proteins such as Bad, termed sensitizers, triggers apoptosis by binding to anti-apoptotic proteins and displacing the activator BH3-only proteins.

ABT-737 is a rationally designed small molecule that binds with high affinity to Bcl-2 and Bcl-xL but not Mcl-1, and antagonizes their anti-apoptotic function.[11,12] ABT-737 has been shown to reverse acquired paclitaxel resistance in breast cancer cell lines.[13] Combined with rapamycin, ABT-737 enhances the radio sensitivity of non-small cell lung tumors.[14] The combination of ABT-737 and a MEK inhibitor leads to tumor regression in different mouse tumor models of KRAS mutant cells.[15] In human myeloid leukemia cell lines and xenograft models, ABT-737 synergizes with inhibitors of the PI3K/AKT/mTOR pathway.[16] Navitoclax (ABT-263), an orally available variant of ABT-737, is currently being evaluated in phase 2 clinical trials. Despite promising preliminary data, thrombocytopenia limits the ability to raise drug concentrations. Since then, ABT-199, a selective Bcl-2 inhibitor with no activity on Bcl-xL, has been shown to achieve anti-tumor activity as a single agent in chronic lymphocytic
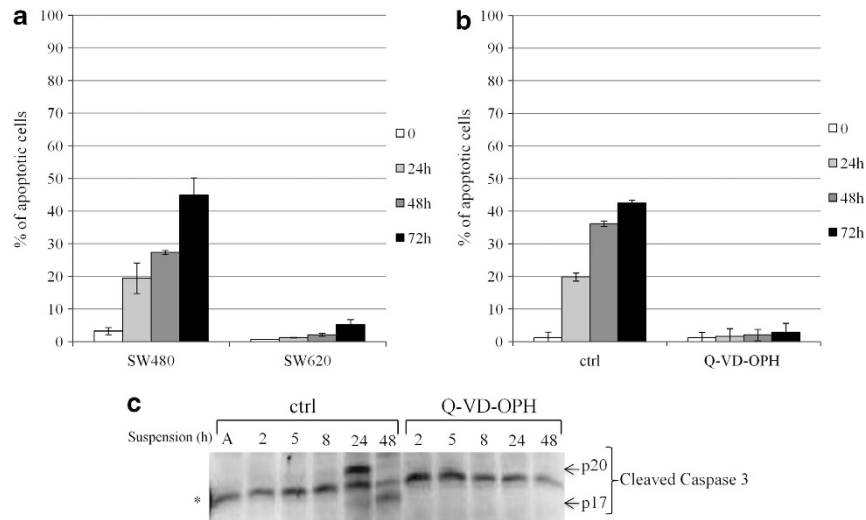
Figure 1 Anoikis sensitivity in SW480 and SW620 cells. (**a**) Time course of apoptotic cell death in suspended SW480 and SW620 cell populations. (**b**) Time course of apoptosis in suspended SW480 cells cultured without (ctrl) or with the pan-caspase inhibitor Q-VD-OPH (10 μM). (**c**) Kinetics of caspase-3 activation in adherent (A) or suspended SW480 cells cultured without (ctrl) or with Q-VD-OPH (10 μM) detected by means of western blot with an anti-cleaved-caspase-3 antibody (p20 and p17 fragments). The asterisk denotes a nonspecific band. For all graphs in all figures, error bars indicate S.D. of at least three independent experiments

leukemia while sparing platelets.[17] The BH3-mimetic ABT-737 and related compounds act as single agents to induce apoptosis only in cancer cells that are dependent on Bcl-2 or Bcl-xL for survival.[18,19] This dependence appears to correlate with seques-tration of activator BH3-only proteins by the anti-apoptotic proteins and such cells have been defined as 'primed for death'. BH3 mimetics, by displacing the activator BH3-only proteins from Bcl-2 or Bcl-xL, allow their interaction with and activation of Bax or Bak. In cells where Bcl-2 and Bcl-xL are not primed, exposure to chemotherapeutic agents has often been shown to induce the expression of activator BH3-only proteins leading to a synergy between these agents and BH3 mimetics.

It has been reported that when cultured in suspension, some anoikis-sensitive cells induce the expression of the BH3-only proteins B-cell lymphoma 2 interacting mediator of cell death (Bim)[20] or Bmf.[21] We therefore reasoned that a parallel could be drawn with the effects of chemotherapeutics and investigated whether, when they are detached, the cell sensitivity to BH3 mimetics is modified. To this end, we used a pair of isogenic colon carcinoma cell lines: SW480 derived from a primary Duke's stage B colon carcinoma, and SW620 derived from a mesenteric lymph node metastasis in the same patient.[22] SW480 cells are sensitive to anoikis, whereas SW620 cells are resistant. Bim was found to be upregulated in suspension in SW480 cells and to mediate, at least in part, their sensitivity to anoikis. Most interestingly, both cell lines died in the presence of ABT-737 when cultured in suspension, whereas they were resistant to this compound under adherent conditions. Thus, detached colon cancer cells are primed for death, whether or not they are sensitive to anoikis, and compounds such as ABT-263 or ABT-199 might therefore have anti-metastatic properties.

Results

SW480 cells are sensitive to anoikis unlike SW620 cells. The time course level of anoikis was compared in SW480 and SW620 cells following culture in suspension. As shown in Figure 1a, the percentage of SW480 apoptotic cells increased with time. In contrast, apoptosis in SW620 cells was barely detectable. In order to check whether SW480 cell death was due to classical apoptosis, cells were cultured in the presence or absence of the pan-caspase inhibitor Q-VD-OPH. The results shown in Figure 1b indicate that this inhibitor fully protected SW480 cells against detachment-induced cell death. We also monitored caspase-3 activation in suspended SW480 cells by the detection of the p20 (intermediate) and p17 (mature) fragments of the active caspase through western blot analysis. The p20 fragment and some p17 fragment were detected at 24 h (Figure 1c) and by 48 h caspase-3 was fully mature. The presence of Q-VD-OPH totally abrogated caspase-3 activation (Figure 1c).

Anoikis has been ascribed to the intrinsic[23] or to the extrinsic[24,25] apoptotic pathway. We therefore studied in kinetics the cleavage and thus the activation of caspase-8 and caspase-9. As can be seen in Figure 2a we detected a faint cleavage band for both caspases as soon as 5 h of culture in suspension. In order to further test which initiator caspase was at the apex of the apoptotic signaling, we used two clones of SW480 cells stably transfected with an expression plasmid encoding for Bcl-2.[26] Both clones were resistant to anoikis (Figure 2b) and, most importantly, activation of all caspases was abrogated in these clones (Figure 2c) indicating that the activation of caspase-8 shown in Figure 2a occurs down-stream of mitochondrial events. To further document that the extrinsic pathway was not involved, SW480 cells were stably transfected with an expression plasmid encoding a dominant-negative form of Fas-associated death domain (FADD) (FADD-dn) and three clones were obtained (Figure 2d). All the clones showed resistance to TRAIL-induced apoptosis, as illustrated in Figure 2e for clone 4. However, all clones were sensitive to anoikis, as illustrated in Figure 2f for clone 4, ruling out the extrinsic pathway.
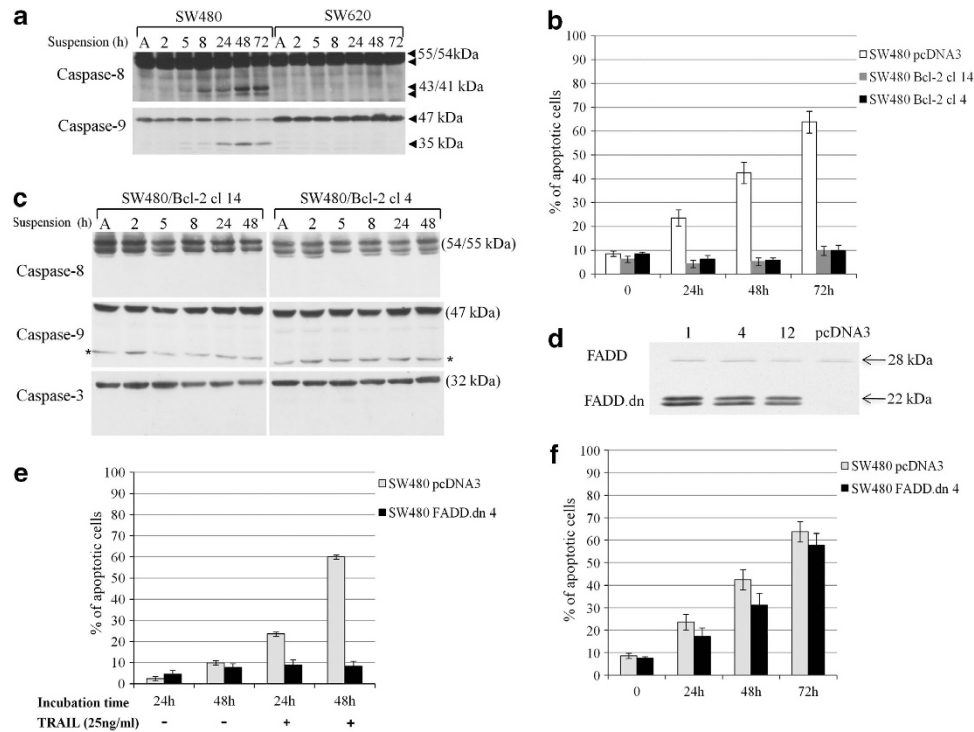
Figure 2 Anoikis in SW480 cells is initiated at the mitochondria. (a)Kinetics of initiator caspase activation in SW480 and SW620 cell lines cultured in suspension. Full-length (55/54 kD) or cleaved(43/41 kD) caspase-8 and full-length (47 kD) or cleaved (35 kD) caspase-9 are detected. (b) Percentage of anoikis in empty vector or Bcl-2-transfected SW480 clones. (c) Lack of detection of caspase activation in Bcl-2-transfected clones during the culture in suspension. Nonspecific band is represented by *. (d) Cell extracts of SW480 clones transfected with FADD.dn (clones 1,4 and 12) or with empty vector (pcDNA3) were blotted as described in Materials and Methods with an anti-FADD antibody (e, f) SW480 cells transfected with empty vector (SW480 pcDNA3) or expressing FADD.dn (clone 4) were either cultured in the presence of recombinant TRAIL (25 ng/ml) (e) or cultured in suspension (f), and apoptosis was quantified at various times

SW620 cells' resistance to anoikis is not due to epithelial–mesenchymal transition. Epithelial–mesenchymal transition (EMT) is not only a key event for epithelial cells to acquire a motile phenotype but it also allows cancer cells to avoid anoikis.[27] During the EMT process, E-cadherin expression is lost, whereas N-cadherin expression is upregulated, a process referred to as the 'cadherin switch'.[28] Both the loss of E-cadherin[29] and the acquisition of N-cadherin[30] expression appear important for the generation of survival signals. SW480 cells display an epithelial morphology, whereas SW620 cells have a rounded or fibroblast-like morphology reminiscent of cells having undergone EMT.[31] The expression of various epithelial and mesenchymal markers was therefore studied in both cell lines. As shown in Figure 3, the expression of E-cadherin was slightly diminished in SW620 cells compared with SW480 cells but cytokeratin 18, another epithelial marker was greatly increased in SW620 cells. Concerning mesen-chymal markers, vimentin was present in both cell lines but neither N-cadherin nor fibronectin was expressed, whereas both were found in murine myeloblasts. Thus, SW620 cells do not seem to be of mesenchymal nature.

The BH3-only protein Bim has a role in SW480 cell death due to anoikis. Given that mitochondrial apoptosis is governed by members of the Bcl-2 family, the kinetic of expression of several of these proteins was studied in both SW480 and SW620 cell lines following their culture in
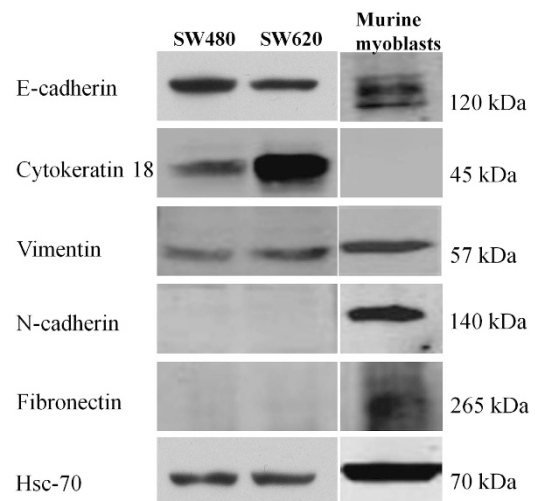
Figure 3 Expression of various epithelial or mesenchymal markers in SW480 and SW620 cells. Cell extracts were blotted with various antibodies recognizing epithelial (E-cadherin, Cytokeratin 18) or mesenchymal (vimentin, N-cadherin, fibronectin) markers. Murine myeloblasts were used as a positive control for mesenchymal cells

suspension. As shown in Figure 4a, the anti-apoptotic Bcl-2 and Bcl-xL were expressed at comparable levels in both cell lines and their expression did not vary during the culture in

Figure 4 Study of the expression of Bcl-2 protein family in SW480 and SW620 cells cultured in suspension. (**a**) Time course analysis of the expression of several mem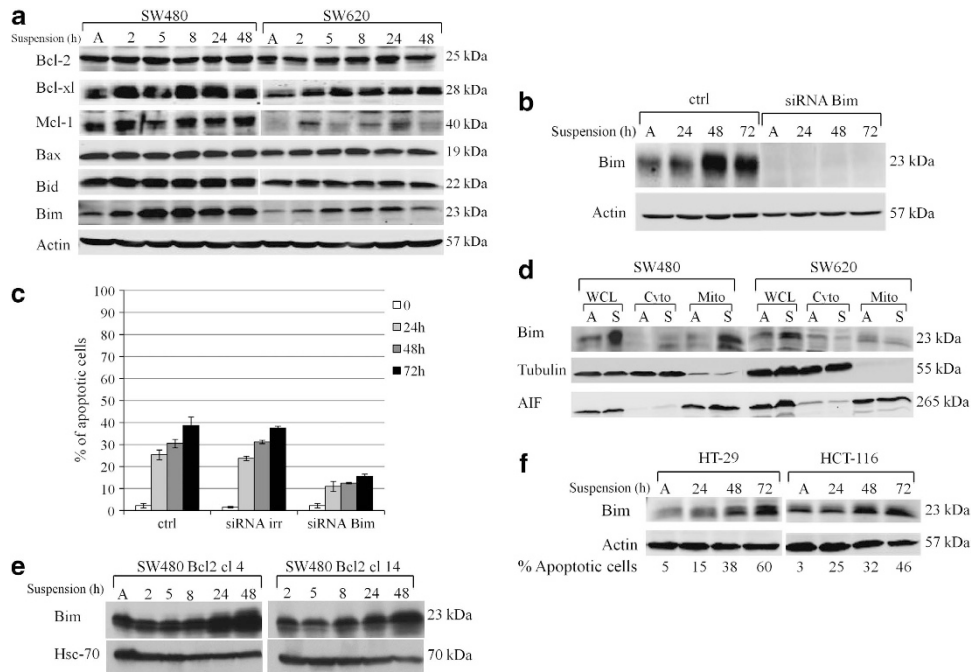bers of the Bcl-2 protein family analyzed by means of western blot in adherent (A) or suspended SW480 and SW620 cells. (**b**) Bim knockout efficiency in adherent (A) or suspended SW480 cells treated (siRNA Bim) or not (ctrl) with a specific Bim siRNA monitored by means of western blot using a specific anti-Bim antibody. (**c**) Percentage of apoptosis in SW480 cell populations cultured in suspension either untransfected (ctrl) or following transfection with a specific Bim siRNA (siRNA Bim) or siRNA to an irrelevant target (siRNA irr). (**d**) Analysis of Bim expression in whole cell lysates and in the cytosolic (cyto) or membrane/organellar (mito)-enriched fractions in adherent cells (A) or after 24 h of suspension (S). (**e**) Time course analysis of the expression of Bim in SW480 overexpressing Bcl-2 (SW480 Bcl-2 4, SW480 Bcl-2 14) either adherent (A) or cultured in suspension. (**f**) Bim expression in malignant colorectal HT-29 and HT-116 cell lines cultured in suspension

suspension. Mcl-1 expression did not change during the culture in SW480 cells in contrast with a recent report that found a substantial reduction of Mcl-1 expression in NIH3T3 cells during anoikis.[32] Overall, in our study, the anoikis-resistant SW620 cells expressed less Mcl-1 than SW480 cells and, for unknown reasons, its level of expression appeared sometimes to fluctuate during the culture. This result might be due to the very short Mcl-1 half-life (40 min).[33] Among the pro-apoptotic proteins, Bax and Bid expression levels were stable in suspended cells. In contrast, the expression of the BH3-only protein Bim greatly increased during the culture in suspension of SW480 cells and within 5 h after cell detachment. Of note is the fact that mostly the Bim-EL variant[34] was detected in these cells and is referred to as Bim throughout the study. Bim expression was increased in SW620 but to a lesser degree.

We abrogated Bim expression in SW480 cells with a specific small-interfering RNA. As shown in Figure 4b, Bim expression was fully inhibited by this treatment throughout the culture in suspension. Lack of Bim was found to greatly but not totally protect SW480 cells from anoikis (Figure 4c). Therefore, we can conclude that Bim is involved in the sensitivity of SW480 cells to anoikis. Bmf has been implicated in the death due to anoikis of several cell types.[21,35,36] However, we could not detect Bmf by western blot analysis in any of the two cell lines (not shown).

In healthy cells, Bim-EL can be sequestered to the microtubule-associated dynein motor complex through its binding to LC8.[37] Certain apoptotic stimuli disrupt the interaction between LC8 and the dynein motor complex, thereby freeing Bim and thus enabling its translocation to the mitochondria. We therefore sought to determine whether, in addition to Bim level in suspended cells, there might also be differences in its localization in SW480 cells compared with SW620 cells. Microtubules depolymerize within a few minutes at room temperature and at 4 °C and therefore cannot be isolated by centrifugation. We utilized another approach with the use of the 'cell fractionation kit' as described in Material and Methods. The cell extracts that we obtained were clean, as monomeric tubulin was found exclusively in the cytosolic protein-enriched fraction, whereas AIF, a mitochondrial protein, was detected exclusively in the membrane and organellar protein-enriched fraction (Figure 4d). Interestingly, Bim in adherent SW480 cells was found mainly in the membrane and organellar protein-enriched fraction, and upregulated Bim in suspended cells was detected in this same fraction. Therefore, the increased amount of Bim that we find in detached SW480 cells is largely localized in the mitochondria. In contrast, Bim was found to distribute roughly equally in the cytosolic and membrane protein-enriched fractions in adherent and suspended SW620 cells. These results suggest that there might be a less efficient translocation of Bim to the mitochondria in SW620 cells.

We then tested the two Bcl-2 expressing SW480 clones that we found to be resistant to anoikis. Both clones upregulated Bim when cultured in suspension (Figure 4e) in the same order of magnitude compared with the parental cells (Figure 3a).
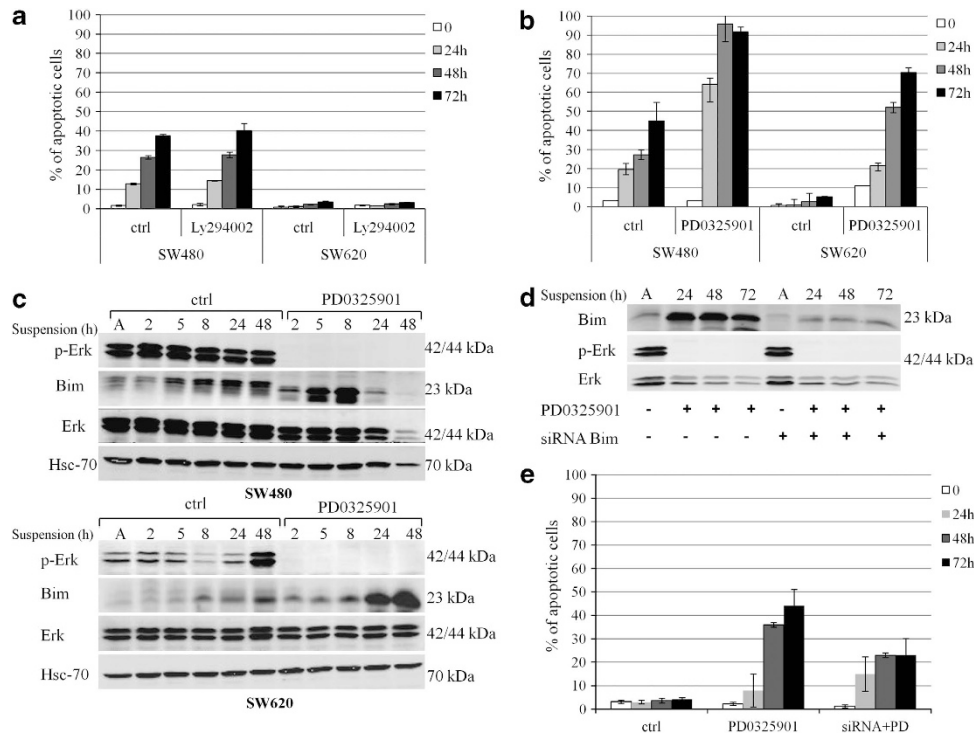
Figure 5 Regulation of Bim protein expression. (**a**) Percentage of apoptosis in suspended SW480 and SW620 cells cultured in the presence (Ly294002) or absence (ctrl) of the PI3K inhibitor Ly294002 (10 μM). (**b**) Percentage of apoptosis in suspended SW480 and SW620 cells cultured in the presence (PD0325901) or absence (ctrl) of the MEK inhibitor PD0325901 (10 μM). (**c**) Western blot analysis of the expression of p-ERK, Bim and total ERK in adherent (A) or suspended SW480 (upper panel) and SW620 (lower panel) cells cultured in the presence (PD0325901) or absence (ctrl) of PD0325901 (10 μM). Anti-Hsc-70 was used as a loading control. (**d**) Western blot analysis of Bim expression in PD0325901-treated SW620 cells incubated with or without Bim siRNA. (**e**) Time course of anoikis level in PD0325901-treated SW620 cells incubated or not with Bim siRNA

Therefore, the sensitivity of SW480 cells to anoikis is not solely dictated by the level of expression of Bim but by the ratio of Bim to Bcl-2.

Finally, we used two other malignant colorectal cell lines to check whether Bim upregulation in suspended cells was a general feature. As shown in Figure 4f Bim expression was increased in both HT-29 and HCT-116 cells during the culture in suspension, and this was accompanied by a significant cell death due to anoikis.

ERK inhibition in SW620 cells restores Bim expression in detached cells and their sensitivity to anoikis. The expression of Bim is controlled by both transcriptional and posttranscriptional mechanisms.[38] The forkhead-like transcription factor FOXO3a (forkhead box O3a) is a key transcriptional regulator of Bim and its activity is suppressed through its phosphorylation by Akt. As shown in Figure 5a, blocking of the PI3K/Akt pathway did not sensitize SW620 cells to anoikis. Moreover, western blot analysis using an anti-phospho-Akt antibody, revealed very little amount of activated Akt in SW620 cells (not shown).

On the other hand, ERK-mediated phosphorylation targets Bim for ubiquitylation and proteasomal degradation. An MEK inhibitor (PD0325901) was very efficient in sensitizing SW620 cells to anoikis while also increasing the level of cell death in SW480 cells cultured in suspension (Figure 5b). In SW620 cells (Figure 5c), ERK basal activity, as evaluated by western blot analysis of phosphorylated ERK (p-ERK), was low and

increased at the end of the culture, a finding that we repeatedly observed. The MEK inhibitor was very efficient in abrogating ERK activity and this was accompanied by an upregulation of Bim expression starting at 24 h correlating with anoikis observed at these time points.

We silenced the expression of Bim by RNA interference in suspended SW620 cells cultured in the presence of PD0325901 (Figure 5d). As shown in Figure 5e, extinction of Bim expression decreased the percentage of apoptosis by about half at 72 h of PD0325901 treatment indicating that Bim is involved in the sensitization to anoikis by this compound. These data also indicate that additional mechanisms are responsible for the remaining cell death. Surprisingly, ERK activity was high and sustained in control SW480 cells throughout the culture in suspension (Figure 5c) but did not prevent the upregulation of Bim expression in control SW480 cells starting at 5 h. MEK inhibition induced a stronger augmentation of Bim expression (Figure 5c). In these conditions, Bim electrophoretic mobility was increased due to the fact that Bim is dephosphorylated. Bim was not detectable at late time points, an observation that we believe to be linked to the very high death rate, which affects the quality of the cell extracts. Likewise, total Erk and Hsc-70, the loading control, were diminished at these late time points.

ABT-737 sensitizes both SW480 and SW620 cells to anoikis. The upregulation of Bim in SW480 cells cultured in suspension led us to test whether, as has been shown for
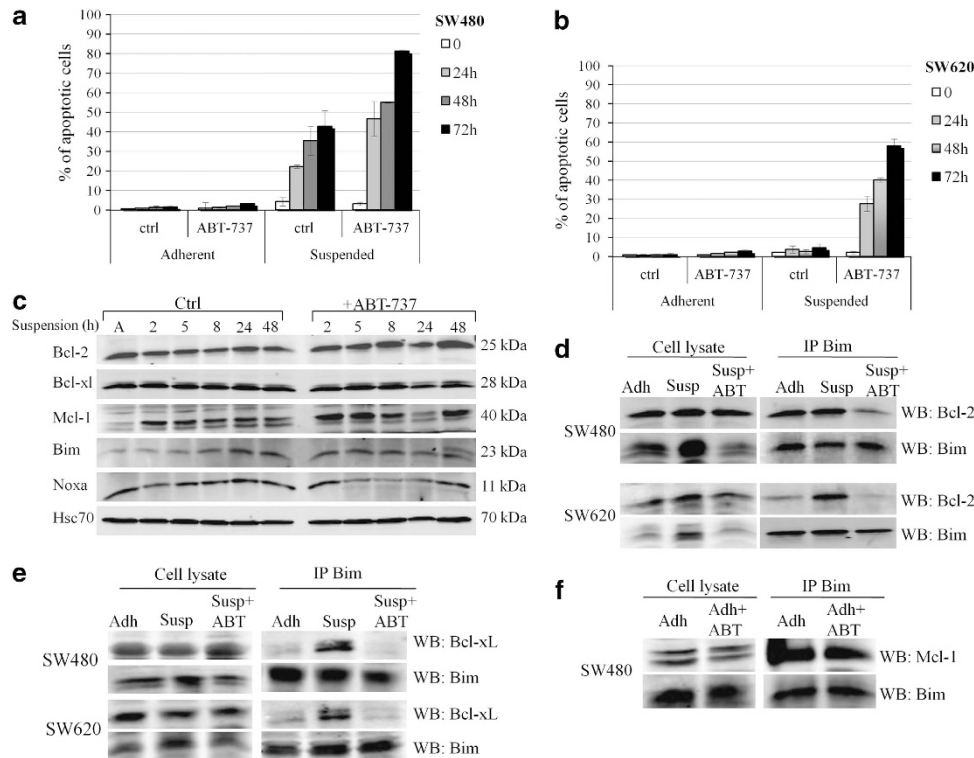
Figure 6 Analysis of SW480 and SW620 cell sensitivity to the BH3-mimetic ABT-737. (**a**, **b**) Percentage of apoptosis in adherent or suspended SW480 (**a**) or SW620 (**b**) cells cultured in the presence (ABT-737) or absence (ctrl) of ABT-737 (1 μM). (**c**) Analysis of the Bcl-2 family member expression in SW620 cells during the culture in suspension in the presence or absense of ABT-737. (**d**) SW480 (upper panels) and SW620 (lower panels) cells either adherent (Adh) or suspended for 24 h in the presence (Susp + ABT) or absence (Susp) of ABT-737 (1 μM) were lysed in CHAPS buffer. Cleared lysates were either analyzed directly (cell lysate) or following immunoprecipitation with anti-Bim antibody (IP Bim) and immunoblotted (WB) for Bcl-2 and Bim as described in Materials and Methods. (**e**) Cells were treated as in **d** and analyzed for Bcl-xL co-immunoprecipitation. (**f**) Adherent SW480 cells cultured in the presence or absence of ABT-737 for 24 h were lysed as in **d** and analyzed for Mcl-1 co-immunoprecipitation

numerous chemotherapeutic drugs used together with Navitoclax,[39] a synergy could be found between the suspended state and ABT-737. As shown in Figure 6a, adherent SW480 cells were totally resistant to ABT-737 (1 μM). However, in suspended cells, the presence of ABT-737 was found to greatly increase the level of anoikis. The same type of experiments were performed in SW620 cells (Figure 6b). Again, adherent cells were resistant to ABT-737. Surprisingly, SW620 cells cultured in suspension, despite their resistance to anoikis, died in significant numbers in the presence of ABT-737. We therefore checked whether the presence of ABT-737 could have modified the expression of some Bcl-2 family members in these cells. As shown in Figure 6c, this was not the case. These observations are particularly important concerning Noxa, as this Mcl-1 inhibitor has been reported to be induced by ABT-737 treatment in a glioma cell line.[40]

Cells sensitive to ABT-737 are supposed to be primed for death with Bcl-2 and/or Bcl-xL complexed to activator BH3-only proteins.[41] Immunoblots of Bim and Bcl-2 or Bcl-xL were therefore performed in whole cell lysates and in anti-Bim immunoprecipitates in SW480 and SW620 cell lines. As shown in Figure 6d, Bcl-2:Bim complexes were detected in both cell lines cultured in suspension. The formation of these complexes was abrogated in the presence of ABT-737, indicating that this compound displaced Bim from Bcl-2 (Figure 6d). Most importantly, Bcl-xL:Bim complexes were

also detected in suspended SW480 and SW620 cells and displaced by ABT-737 (Figure 6e). Bcl-2:Bim complexes were also found in adherent SW480 cells (Figure 6d) despite their lack of sensitivity to ABT-737 in these culture conditions. It is possible that, in the absence of Bim upregulation, the amount of such complexes is too low to offset the anti-apoptotic function of Mcl-1[42], which is highly expressed in SW480 cells (Figure 3a). We performed immunoprecipitations of Bim in adherent SW480 cells treated or not with ABT-737, which revealed the presence of Bim:Mcl-1 complexes in both conditions (Figure 6f). Therefore, we believe that, as Bim level is low in adherent SW480 cells, Mcl-1 is able to capture the small amount of protein displaced from Bcl-2/Bcl-xL by ABT-737.

Discussion

Anoikis is by essence an anti-metastatic process and resistance to this form of cell death correlates with cancer aggressiveness.[4]

In this study, we used the SW480 and SW620 cell lines that have been validated as a model of colon cancer progression from primary tumor (SW480) to metastatic cells (SW620)[31], and show that during this progression the tumor cells have acquired an anoikis-resistant phenotype. EMT has been shown to be an important phenomenon for the acquisition of anoikis resistance[43]; however, we did not find any evidence

that this process had occurred in SW620 cells despite their suggestive fibroblast-like morphology. It remains possible that these metastatic cells have undergone transient EMT followed by mesenchymal–epithelial transition once they have colonized their secondary site, as has been suggested in invasive colon cancer.[44]

Among the pro-apoptotic proteins tested, Bim was the only one to be highly upregulated in suspended SW480 cells. Importantly, silencing of Bim expression confirmed that this BH3-only protein was involved in SW480 cell death by anoikis. However, the level of apoptosis went from 38% in control cells to 15% in cells treated with the Bim short interfering RNA (siRNA) indicating that another pathway might be implicated in this remaining cell death. We determined that an implication of Bmf was unlikely.

Most interestingly, when the effect of ABT-737 on suspended cells was evaluated, this compound was found to enhance anoikis not only in SW480 cells, which upregulate Bim in suspension, but also in SW620 cells, which only slightly do so. Several studies have been performed to determine ABT-737 mode of action. One of these studies, in acute myeloid leukemia, indicates that ABT-737 acts by inducing a dissociation of Bcl-2:Bax complexes and induces apoptosis in a Bak-dependent but Bim-independent manner.[45] However, we have been unable to detect such complexes in either cell line in adhesion or in suspension (not shown). On another hand, in both cell lines cultured in suspension, Bcl-2:Bim and Bcl-xL:Bim complexes were immunoprecipitated and these complexes were disrupted by ABT-737. Our data are therefore more in accordance with results obtained in chronic lymphocytic leukemia, where liberation of Bim from Bcl-2 by ABT-737 leads to Bax activation.[19] Thus, Bim occupation of Bcl-2 and Bcl-xL in suspended cells primed SW480 and SW620 cells for death and ABT-737 acted by freeing Bim. Bcl-2:Bim or Bcl-xL:Bim complexes were hardly detected in adherent SW620 cells, likely explaining why they were resistant to ABT-737. In contrast, adherent SW480 cells displayed Bcl-2:Bim complexes, whereas these cells were not sensitive to ABT-737. Mcl-1 has been shown to be a key determinant of the resistance to ABT-737[45,46], and SW480 cells express high levels of Mcl-1. Therefore, even if Bcl-2:Bim complexes were disrupted by ABT-737 in these adherent cells, the displaced Bim could be bound by Mcl-1, maintaining survival. Indeed, we could detect Mcl-1:Bim complexes in adherent SW480 cells treated or not with ABT-737. Upregulation of Bim in the suspended SW480 cells should allow for more Bcl-2:Bim complexes to be formed. In this case, the amount of Bim freed by ABT-737 would subvert the buffering capacity of Mcl-1, thus rendering the cells sensitive to the BH3-mimetic.

Bim steady-state level is regulated through its ERK1/2-dependent phosphorylation, which targets it for degradation by the proteasome.[47] Inhibition of MEK was found to upregulate Bim expression in both cell lines and to sensitize SW620 cells to anoikis. Thus, SW620 cells in suspension are sensitive to Bim-induced apoptosis provided that this BH3-only protein is present in sufficient amounts. However, it appears unlikely that the ERK pathway is solely responsible for the lack of Bim upregulation in suspended SW620 cells, as we did not observe a strict negative correlation between the p-ERK level and Bim expression. Indeed, ERK activity is low in these cells and increased only at later time points. Given our results that the PI3K–Akt pathway is also not involved, it is likely that another pathway, which remains to be identified, is also at play in the regulation of Bim expression.

Mcl-1 level, high and stable during the culture in suspension of SW480 cells was lower and sometimes fluctuating in SW620 cells. It is interesting to note that Mcl-1 degradation has been shown to be necessary for anoikis induction in some models. This is clearly not the case in SW480 cells. The short half-life of Mcl-1 has been ascribed to its phosphorylation by GSK-3 followed by its ubiquitination and degradation by the proteasome.[48] GSK-3 is inhibited by the PI3K–Akt pathway. We found very little Akt activity in SW620 cells, and GSK-3 might therefore be responsible for the reduced amount of Mcl-1 expressed by SW620 cells. In any case, low level of Mcl-1 would be expected to sensitize SW620 cells to anoikis, which is not what we observed.

In conclusion, whether or not they undergo anoikis, suspended colon cancer cells are sensitive to the BH3-mimetic ABT-737. This indicates that cancer cells that have detached from the extracellular matrix might go through a transient window where they can be killed by compounds such as Navitoclax or ABT-199, which would give anti-metastatic properties to these agents.

Materials and Methods

Cell lines and reagents. SW480 and SW620 human colon carcinoma cell lines were obtained from the American Type Culture Collection (ATCC). They were routinely maintained in Dulbecco's minimal essential medium (DMEM) (Gibco, Saint Aubin, France) supplemented with 10% heat-inactivated fetal bovine serum, 1% sodium pyruvate, 0.1 mg/ml streptomycin, 100 U/ml penicillin at 37 °C in a 5% CO_2-humidified atmosphere. For the cultures in suspension, 2 units/ml of dispase (Roche, Meylan, France) was added to the culture medium to obtain single cell suspensions. Dispase is a bacterial neutral protease with mild proteolytic activity, which is mainly used for the isolation and routine passaging of primary cells. It can also detach adherent cells from the culture vessel[49] by digesting the extracellular matrix[50] and dissociating cellular aggregates.[51] Dispase is not inactivated by serum and, given its lack of toxicity, is a very efficient mean for keeping adherent cells in unicellular suspension.

TRAIL sensitivity of SW480 clones was tested by culturing the cells in the presence of Flag-tagged recombinant TRAIL (25 ng/ml) (Alexis Biochemicals, Villeurbanne, France) and anti-Flag (M2) antibody (Sigma Aldrich, Lyon, France) for various times.

Stock solutions of Q-VD-OPH (Biovision, Nanterre, France), Ly294002 (Calbiochem, Darmstadt, Germany) and PD0325901 (Sigma Aldrich) were prepared in DMSO at 10 mM and used at 10 μM final concentration. ABT-737 (Selleckchem, Souffelweyersheim, France) was also prepared in DMSO at 10 mM and used at 1 μM final concentration.

The following antibodies were used: anti-Bax (N-20, Santa Cruz Biotechnology, Inc, Heildelberg, Germany), anti-Bid (R&D Systems, Lille, France), anti- cleaved caspase-3 (Cell Signaling, Saint Quentin en Yvelines, France), anti-Hsc70 (B-6, Santa Cruz Biotechnology, Inc), anti-Mcl-1 (S-19, Santa Cruz Biotechnology, Inc), anti-Bcl-2 (H-100, Santa Cruz Biotechnology, Inc), anti-Bcl-xL (BD Transduction Laboratories, Le Pont de Claix, France), anti-Bim (Cell Signaling), anti-pErk (Cell Signaling), anti-Erk total (Cell Signaling), anti-actin (MBL, Nanterre, France) and anti-FADD (BD Transduction Laboratories).

Flow cytometry analysis of apoptotic cells. Suspended cells maintained in culture from 24–72 h were washed in PBS (phosphate-buffered saline), fixed in 70% cold ethanol and stored at − 20 °C for at least 12 h. After two washes in PBS, cells were resuspended and stained in 0.5 ml of PBS containing 50 μg/ml propidium iodide. The fluorescence intensity of propidium iodide was assessed with a FACSCalibur flow cytometer (Becton Dickinson, Le Pont de Claix, France) and analyzed using the CellQuest (Becton Dickinson) software. The percentage of cells with apoptotic nuclei, distinguished by their hypodiploid DNA content (sub-G0/G1 peak),[52] was assessed for each histogram.

Western blotting and immunoprecipitation. Cells were lysed with ice-cold NP40 lysis buffer (30 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 10 mM NaPP and 1% NP40) containing 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate (Na_3VO_4) and 1/100 protease cocktail inhibitors (Sigma, Lyon, France). All lysates were clarified by centrifugation at $15\,000 \times g$ for 15 min at 4 °C. Protein concentrations were assessed using the Bradford assay (BioRad, Hercules, CA, USA). Proteins amounting to 50 μg were prepared in loading Laemmli's buffer (60 mM Tris-HCL pH 6.8, 0.18 M β-mercaptoethanol, 2% SDS, 10% glycerol and 0.005% bromophénol blue), boiled for 5 min, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on ice for 1.5 h to polyvinylidenedifluoride membrane (Amersham Hybond-LFP, GE healthcare, Velizy-Villacoublay, France). After blocking for at least 1 h in Tris-buffered saline supplemented with 5% BSA and 0.5% Tween-20 (TBS-T), membranes were probed with the appropriate primary antibodies in TBS-T overnight. Proteins were visualized by using Lycor secondary antibodies and Odyssey detection material.

For immunoprecipitation experiments, cells were lysed with CHAPS lysis buffer (30 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 10 mM NaPP and 1% CHAPS) containing 1 mM phenylmethylsulfo-nylfluoride, 1 mMsodium orthovanadate (Na_3VO_4) and 1/100 protease cocktail inhibitors). Nuclear pellets and debris were removed by centrifugation, at $15\,000 \times g$ for 15 mn at 4 °C. Six milligrams of proteins from cell lysates were incubated for 1 h at 4 °C with the anti-Bim antibodies. Protein-G beads were added to the immune complexes for 45 min and washed five times with ice-cold CHAPS lysis buffer. Purified immunoprecipitates, immobilized on protein-G beads, were mixed with an equal volume of Laemmli's buffer 2x, boiled for 5 min and further analyzed by means of western blot for both Bim and Bcl-2 content.

Small-interfering RNA-mediated silencing of Bim. In 3 ml of culture medium, 3×10^5 cells were transfected with Bim siRNA or irrelevant siRNA (Ambion Life Technologies, Saint Aubin, France). Each siRNA was used at 20 nM final concentration. INTERFERin (20 μl, Polyplus transfection, Ozyme, Saint Quentin en Yvelines, France) was incubated with siRNA duplex in 800 μl of DMEM without serum for 20 min at room temperature. The mixture was then added to the cells, which were transferred to culture plates and incubated at 37 °C. Seventy-two hours after transfection, cells were detached with culture medium containing 2 units/ml of dispase, cultured in this medium for 24, 48 or 72 h and the percentage of apoptotic cells was quantified as described above. Extinction of Bim expression by the Bim siRNA was monitored by means of western blot throughout the culture in suspension.

Stable transfection of FADD.dn in SW480 cells. The pcDNA3/FADD.dn vector encodes for a truncated form of FADD protein deleted of its two DED domains and thus unable to recruit caspase-8. SW480 cells were transfected 5 μg of either pcDNA3/FADD.dn or pcDNA3 empty vector with the use of JetPei (Polyplus transfection). Transfected cells were selected with neomycin (400 μg/ml) and then cloned.

Cell fractionation. We used the 'cell fractionation kit' (catalog no. 9038) from Cell Signaling Technology according to manufacturer's instructions. This methodology is detergent-based[53] and is performed on ice. Cell pellet is resuspended in a first, digitonin-based, buffer for 5 mn followed by a centrifugation at $500 \times g$. The supernatant is the cytosolic protein-enriched fraction. The pellet is resuspended in a second, triton-based buffer for 5 mn and centrifuged at $8000 \times g$. The supernatant is the membrane and organellar protein-enriched fraction, which contains, among others, mitochondria-associated proteins. The remaining pellet, which we did not use, contains the actin cytoskeleton and the nuclear proteins. Given that microtubules depolymerize within minutes on ice, tubulin and all associated proteins, including dynein motor complex-bound Bim for our purpose, end up in the cytosolic fraction.

Conflict of Interest

The authors declare no conflict of interest.

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