



## **ABT-263, an Inhibitor of Bcl-2 Family Antiapoptotic Proteins, Sensitizes Prometaphase-Arrested HeLa Cells to Apoptosis Induced by Mild Hyperthermia**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author JRP designed the study, planned and carried out some experiments and wrote the manuscript. Authors LX and JRP synthesized 1NM-PP1. Authors RLL, SS and DO planned and carried out experiments. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** We have previously shown that mild hyperthermia (39-42°C) rapidly induces apoptosis in H-HeLa cells arrested in mitosis but not in interphase cells and not in mitotic-arrested cells of other HeLa strains such as HeLa S3. Our goal was to test the idea that this sensitivity to hyperthermia is due to down-regulation of Bcl-2 family antiapoptotic proteins during mitosis.

**Methodology:** HeLa S3 cells were arrested in mitosis with spindle poisons and simultaneously treated with mild hyperthermia (42°C) and ABT-263 (Navitoclax), an inhibitor of Bcl-2 and other anti-apoptotic proteins. Apoptosis was observed microscopically and confirmed by observation of procaspase 3 cleavage using western immunoblotting.

**Results:** Treatment of prometaphase-arrested HeLa S3 cells with mild hyperthermia (42°C) and as little as 200 nM ABT-263 induces apoptosis in the great majority of cells within 2 hr, as judged by blebbing of plasma membranes. The effect is not seen with interphase cells and is blocked by caspase inhibitors z-VAD-fmk and z-DEVD-fmk.

**Conclusion:** Our results show that inhibition of Bcl-2 family antiapoptotic proteins sensitizes prometaphase-arrested HeLa cells, but not interphase cells, to apoptosis induced by mild hyperthermia. An open question is why are prometaphase-arrested cells more susceptible than

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interphase cells? If the features of mitotic-arrested cells that make them sensitive could be identified and mimicked in interphase cells by appropriate drug treatments, it may be possible to combine systemic chemotherapy with local hyperthermia to provide an effective new treatment for tumors.

**Keywords:** *ABT-263; apoptosis; Bcl-2; HeLa; hyperthermia; mitosis; metaphase-arrest.*

## 1. INTRODUCTION

Apoptosis is a form of programmed cell death by which the body eliminates cells that are unneeded, unwanted or irreparably damaged. It plays an important role in embryological development, in defense against disease, and in surveillance for cells that have the potential to become cancerous due to damage from radiation, chemical insults or infections. Apoptosis is distinguished by activation of specific proteases called caspases and leads to destruction of cells in a way that does not trigger inflammation or autoimmunity [1-3].

Apoptosis can be initiated in two different ways. In the extrinsic pathway, the cell responds to external signals via “death receptors” on the cell surface, whereas in the intrinsic pathway apoptosis is triggered by cell stress [4-6]. In either case, activation of caspases produces distinctive biochemical and morphological changes characteristic of apoptosis: blebbing of the plasma membrane, fragmentation of the nuclear DNA, hypercondensation and fragmentation of the chromatin, and proteolytic cleavage of various proteins [7-10].

Treatment of cancer with chemotherapy or radiotherapy often involves induction of apoptosis via the intrinsic pathway [4,11-15]. Apoptosis can also be induced by heat treatment (hyperthermia, or thermotherapy) and this has also been used to treat tumors (e.g., [16-21]). The effectiveness of hyperthermia can sometimes be increased by using it in combination with chemotherapy or radiotherapy (e.g., [22-25]).

We have previously reported that apoptosis is rapidly induced when prometaphase-arrested (but not interphase) H-HeLa cells [26] are subjected to mild heat treatment at 39-42°C [27]. This effect was not seen with other HeLa strains tested including for example HeLa S3. These results raised some interesting questions. First, what is the difference between prometaphase-arrested and interphase H-HeLa cells that makes the former more sensitive to hyperthermia?

Second, why do H-HeLa and HeLa S3 behave so differently? These questions are potentially important, because if one could identify the biochemical features of prometaphase-arrested H-HeLa cells that make them sensitive to mild hyperthermia, it might be possible to use appropriate drugs to mimic those features in interphase HeLa cells and in other human cancer cells. In short, it might make possible a new approach to the treatment of human tumors involving systemic chemotherapy combined with localized hyperthermia.

With the goal of answering these questions, we have begun to explore possible roles for Bcl-2 family proteins [28]. These proteins, some of which are proapoptotic (e.g., Bak and Bax) and some of which are antiapoptotic (e.g., Bcl-2 itself, Bcl-xL, Bcl-w and Mcl-1), are mainly involved in controlling the integrity of the mitochondrial outer membrane and thereby regulating the release of cytochrome c, which triggers the intrinsic pathway. Following its release cytochrome c binds to Apaf to form the apoptosome which subsequently activates the caspases that carry out the “execution” of the cell. Antiapoptotic Bcl-2 family proteins are often associated with resistance to chemotherapy, so in recent years much effort has been made to develop inhibitors of these proteins [29-32].

It has been reported that anti-apoptotic proteins like Bcl-2 may be down-regulated by phosphorylation during mitosis [33]. This suggested to us that the differing sensitivity of prometaphase-arrested and interphase H-HeLa cells to hyperthermia [27] may be due to differential activity of Bcl-2 family proteins. Moreover, variations in the activity of these proteins may explain why some HeLa strains are more sensitive to hyperthermia than others.

Here, we have examined the effects of mild hyperthermia on HeLa cells treated with ABT-263, an inhibitor of Bcl-2 and other anti-apoptotic proteins. We find that combined treatment of prometaphase-arrested HeLa cells with mild heat and ABT-263 leads rapidly to apoptosis.

However, the same treatment has little apparent effect on interphase cells.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Media

The Bcl-2 inhibitor ABT-263 (Navitoclax) was purchased from Selleckchem.com or ApexBio, prepared as a 5 mM solution in dimethylsulfoxide (DMSO) and stored at  $-80^{\circ}\text{C}$ . The caspase 3 inhibitor z-DEVD-fmk and the pan-caspase inhibitor z-VAD-fmk were purchased from AdooQ Bioscience, dissolved in DMSO at 10 mM and 50 mM respectively, and stored at  $-20^{\circ}\text{C}$ . Bcl-2 inhibitor ABT-199 (Venetoclax) and Mcl-1 inhibitor S63845 were obtained from Chemgood, dissolved in DMSO at a concentration of 5 mM and stored at  $-20^{\circ}\text{C}$ . Stock solutions of colchicine (2 mg/mL), S-trityl-L-cysteine (50 mM), paclitaxel (2.5 mg/mL) and nocodazole (2 mg/mL) were prepared in DMSO and stored at  $-20^{\circ}\text{C}$ . The ATP analog 1NM-PP1 [34] was synthesized as described in [35], dissolved at 25 mM in DMSO and stored at  $-20^{\circ}\text{C}$ .

RPMI-1640 without glutamine was obtained from Lonza, Walkersville, MD. Newborn calf serum was from Atlanta Biologicals, Penicillin/streptomycin and 200 mM glutamine were from Corning. All other reagents were obtained from Sigma/Aldrich unless otherwise noted.

### 2.2 HeLa Cell Culture, Cell Cycle Synchronization and Mitotic Arrest

Three HeLa strains were used: HeLa-S3, HeLa-MKF-cdk1-as, and H-HeLa(WML). HeLa-MKF-cdk1-as, in which the protein kinase Cdk1 has been made sensitive to the ATP analog 1NM-PP1 [35], was obtained from Dr. Kumiko Samejima, University of Edinburgh. H-HeLa(WML), which is related to but not identical to the H-HeLa used in our previous study [26,27], was obtained from the laboratory of Dr. Ann Palmenberg at the University of Wisconsin-Madison.

HeLa MKF-cdk1-as and H-HeLa(WML) cells were grown as monolayers in T75 flasks at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . Medium consisted of RPMI-1640 supplemented with 5% newborn calf serum (heat inactivated), 100 i.u./mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 2 mM glutamine. HeLa S3 stock cultures were grown in the same medium but were

maintained as spinner cultures ( $2-5 \times 10^5$  cells/mL) at  $37^{\circ}\text{C}$  in 250 mL bottles with Teflon-coated magnetic stirring bars [36]. In some experiments, suspension cultures were arrested in prometaphase by treatment for 16-18 hrs with 0.2  $\mu\text{g}/\text{mL}$  nocodazole or colchicine, or 0.25  $\mu\text{g}/\text{mL}$  paclitaxel. Monolayer cultures were treated similarly, and prometaphase-arrested cells harvested by shake-off. However, in many experiments cultures were first synchronized in S-phase by treatment with 2.5 mM thymidine [37,38]. After 19-24 hrs, monolayer cultures were simply washed twice with sterile 0.9% NaCl solution, placed in fresh medium and further incubated at  $37^{\circ}\text{C}$ . Thymidine-treated cells growing in suspension were pelleted, washed twice with saline, resuspended in fresh medium, and incubated at  $37^{\circ}\text{C}$  again. Nocodazole, colchicine or paclitaxel was added 4-6 hours after removal of thymidine. Cultures with viabilities of 90-95% and mitotic indices of 85-95% were routinely obtained at 16-20 hours after release from thymidine. In the experiment shown in Fig. 7, prometaphase-arrested HeLa MKF-cdk1-as cells were obtained by treating monolayers with 2  $\mu\text{M}$  1NM-PP1 for 20 hrs, washing 3 times with fresh, warm medium containing 0.2  $\mu\text{g}/\text{mL}$  nocodazole, continuing incubation at  $37^{\circ}\text{C}$  in medium containing nocodazole, and then harvesting prometaphase-arrested cells by shake-off.

### 2.3 Treatment of Cell Cultures with Drugs and Hyperthermia

Immediately before treatments with heat and/or drugs, cells in suspension were adjusted to a concentration of  $5-10 \times 10^5$  cells/mL by pelleting and resuspending in a portion of the same medium. 5 or 10 mL aliquots of these cells were treated in 25 mL stoppered Erlenmeyer flasks in a rotary water bath shaker (140 rpm) at the appropriate temperatures. Measured amounts of inhibitors (or DMSO, in controls) were added to the flasks before adding cells. Aliquots were taken periodically to determine the percentages of mitotic, interphase and apoptotic cells or to prepare samples for gel electrophoresis.

### 2.4 Light Microscopy and Observation of Apoptosis

To determine the mitotic index of a culture (the percentage of cells in mitosis), 200  $\mu\text{L}$  of a cell culture was mixed with 200  $\mu\text{L}$  of water containing 20  $\mu\text{g}/\text{mL}$  Hoechst 33342, incubated for 5 min at room temperature to allow for

hypotonic swelling, and fixed by adding 40  $\mu$ L of fixative (3:1, methanol:acetic acid) [39]. Slides were viewed by epifluorescence and scanned on a raster. Every cell that came into view was scored as mitotic or interphase until at least 200 cells had been counted. Apoptotic cells can also be easily distinguished in these samples by their highly condensed and fragmented chromatin and their blebbed plasma membranes. However, in most experiments the percentage of apoptotic cells was determined by simply using a hemocytometer to count the relative numbers of cells with blebbed plasma membranes versus those with normal, rounded appearance. Cells were either counted immediately in fresh unfixed samples or fixed by adding glutaraldehyde (final concentration, 2% (v/v)) and counted within 48 hrs. Both methods gave the same results.

## 2.5 SDS-Polyacrylamide gel Electrophoresis and Western Blotting

Western blotting of total cell lysates used rabbit polyclonal anti-human Caspase 3 (GeneTex) as primary antibody and HRP-conjugated goat anti-rabbit IgG (Santa Cruz) as secondary antibody. For each gel sample,  $2 \times 10^6$  cells were chilled, pelleted, washed twice in ice cold isotonic saline, and lysed at  $2 \times 10^7$  cells/mL in 100  $\mu$ L of cold Resuspension Buffer (RB) (10 mM Hepes-Na+ pH 7.4, 10 mM NaCl, 5 mM MgCl<sub>2</sub> and 0.2% NP-40). Immediately 100  $\mu$ L of final sample buffer [40] and 20  $\mu$ L of 2-mercaptoethanol were added and the sample mixed and boiled 2 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis [40] in minigels (6 cm  $\times$  8 cm) with 4% acrylamide in the stacking gel and 14% acrylamide in the separating gel and subsequently transferred electrophoretically (60V for 90 minutes) to nitrocellulose membranes. Membranes were blocked for 90 min at 23°C with 5% powdered milk in PBS and exposed to the primary antibody (1:500 in 5% milk in PBS) for 3 hr at 28°C. Membranes were then washed three times in 2.5% milk in PBS and exposed to the secondary antibody (1:2000 in 2.5% milk in PBS) for 45 min at room temperature. After washing with PBS, bound secondary antibodies were visualized using enhanced chemiluminescent staining (ECL-Prime, GE Healthcare) and Kodak X-Omat AR X-ray film.

## 3. RESULTS

In previous work, we showed that treatment of prometaphase-arrested H-HeLa cells with mild hyperthermia (39-42°C) led to induction of

apoptosis. However, the same treatment did not induce apoptosis in interphase H-HeLa cells or in prometaphase-arrested cells of other HeLa strains [27]. Why do interphase and mitotic cells differ in their sensitivity to hyperthermia? One possibility is differences in the level or activity of antiapoptotic Bcl-2 family proteins. It has been reported that Bcl-2 may be down-regulated by phosphorylation during mitosis [33]. This suggested that small molecule inhibitors of antiapoptotic Bcl-2-family proteins might make cells more sensitive to hyperthermia-induced apoptosis.

To test this idea, prometaphase-arrested and interphase HeLa cells were treated at 42.0°C in the presence of ABT-263 (Navitoclax), an inhibitor of Bcl-2 and related proteins [29,41]. As judged by observation of plasma membrane blebbing, this treatment appeared to induce apoptosis rapidly in prometaphase-arrested cells, but not in interphase cells.

Although most experiments were done with HeLa S3, similar results were obtained with H-HeLa(WML) and HeLa MKF-cdk1-as. Nocodazole was used for mitotic arrest in all the experiments presented here, but mitotic arrest with paclitaxel, colchicine or S-trityl-L-cysteine gave essentially the same results.

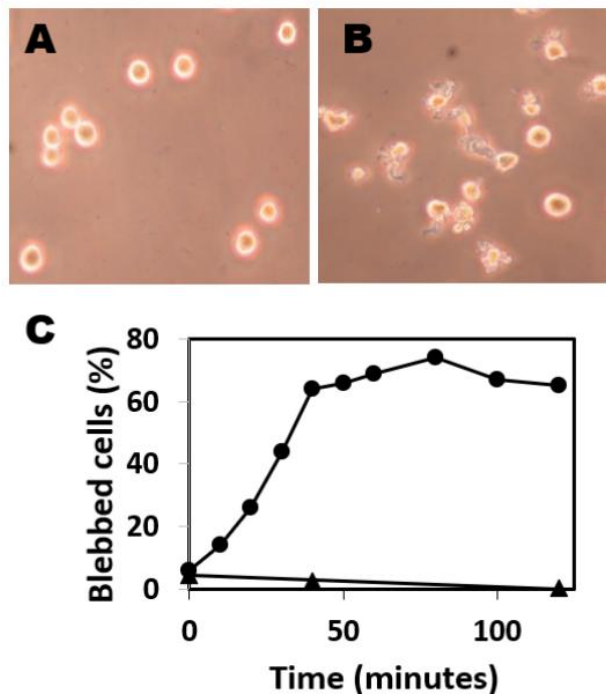
Fig. 1 demonstrates that treatment of HeLa S3 with ABT-263 at 42°C rapidly induces blebbing of the plasma membrane in prometaphase-arrested cells but not in interphase cells. Fig. 1A and B show interphase and prometaphase-arrested cells, respectively, after 40 min of this treatment. Note that many cells in Fig. 1B have blebbed plasma membranes. Fig. 1C shows the time-course of blebbing in the two cases. In this and most experiments, 5  $\mu$ M ABT-263 was used, but lower concentrations suffice. Fig. 2 shows that with prometaphase-arrested HeLa S3 cells, 200-500 nM is sufficient for nearly maximal induction after 2 hr at 42°C.

Fig. 3 presents further evidence that treatment with ABT-263 and mild hyperthermia induces apoptosis only in mitotic cells. In this experiment the mitotic index of the HeLa S3 culture was 70%. Samples at each time point were swollen hypotonically, fixed with methanol:acetic acid, stained with Hoechst 33342, and viewed by epifluorescence microscopy (see Materials and Methods). This procedure allows interphase, mitotic and apoptotic cells to be distinguished

[27]. At least 200 cells were counted for each data point giving a margin of error of about 7%. Note that the number of cells in mitosis falls as the number of apoptotic cells rises, while the percentage of cells in interphase remains constant, within the margin of error.

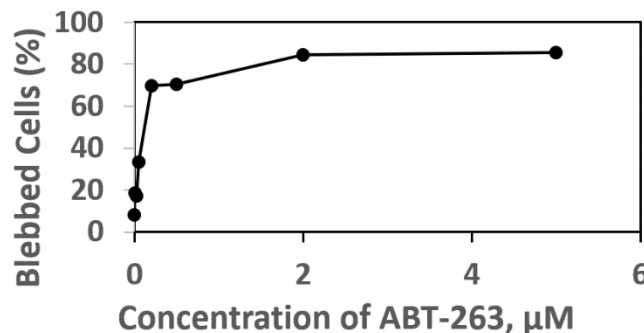
Fig. 4 shows that induction of apoptosis (as judged by blebbing of the cell membrane) requires all three components of the treatment: prometaphase-arrest, ABT-263, and

hyperthermia (42°C). In this experiment, about 60% of the cells arrested in mitosis displayed plasma membrane blebbing after treatment with 5 µM ABT-263 for 2 hr at 42°C, while only about 10% were blebbed following treatment with hyperthermia (42°C) alone and only 8% were blebbed following treatment with ABT-263 alone. With interphase cells, blebbing was observed in only 4% of the cells treated for 2 hr with 5 µM ABT-263 at 42°C, and virtually no blebbing was observed in the other samples.



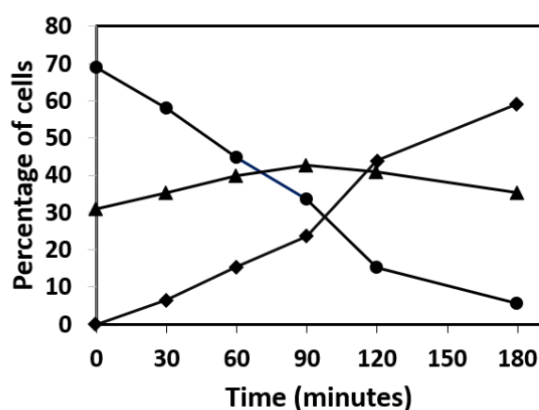
**Fig. 1. Treatment at 42°C in the presence of 5 µM ABT-263 (Navitoclax) induces apoptosis in prometaphase-arrested HeLa S3 cells but not in interphase cells**

Phase-contrast microscopy of (A) interphase cells and (B) prometaphase-arrested cells (mitotic index, 81%) after 40 min treatment. Note blebbing of the plasma membrane, a characteristic of apoptosis. (C) Time course of blebbing for interphase cells (▲—▲) and prometaphase-arrested cells (●—●)



**Fig. 2. The effect of ABT-263 concentration**

Aliquots of a prometaphase-arrested HeLa S3 culture (mitotic index 85%) were treated with various concentrations of ABT-263 and the percentage of blebbed (apoptotic) cells (cf., Fig. 1 B) determined microscopically



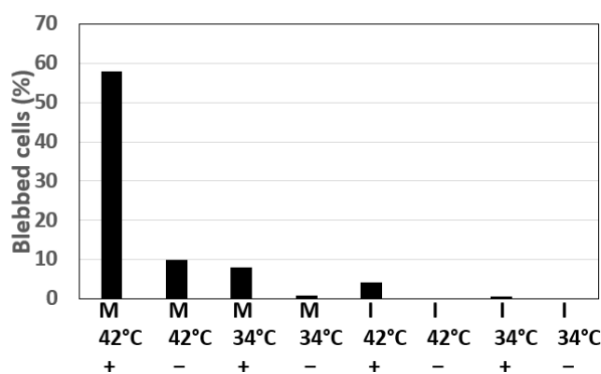
**Fig. 3. Percentage of mitotic, interphase and apoptotic HeLa S3 cells as a function of the time of treatment at 42°C in the presence of 5 μM ABT-263**

Cells in a nocodazole-treated culture (mitotic index, 70%) were viewed by epifluorescence microscopy following hypotonic swelling, fixation, and staining with Hoechst 33342. This method allows mitotic (prometaphase-arrested) (●—●), interphase (▲—▲), and apoptotic cells (◆—◆) to be distinguished. Note that the percentage of mitotic cells falls as the percentage of apoptotic cells increases, but the percentage of interphase cells remains fairly constant

Further experiments confirm that the combination of heat treatment and ABT-263 indeed induces apoptosis and that plasma membrane blebbing is a reliable indicator of that. Fig. 5 shows that the plasma membrane blebbing induced by treatment with ABT-263 at 42°C is blocked by the pan-caspase inhibitor zVAD-fmk and the caspase 3 inhibitor zDEVD-fmk [42]. The western blot in Fig. 6 shows that caspase 3 is cleaved and activated during the treatment.

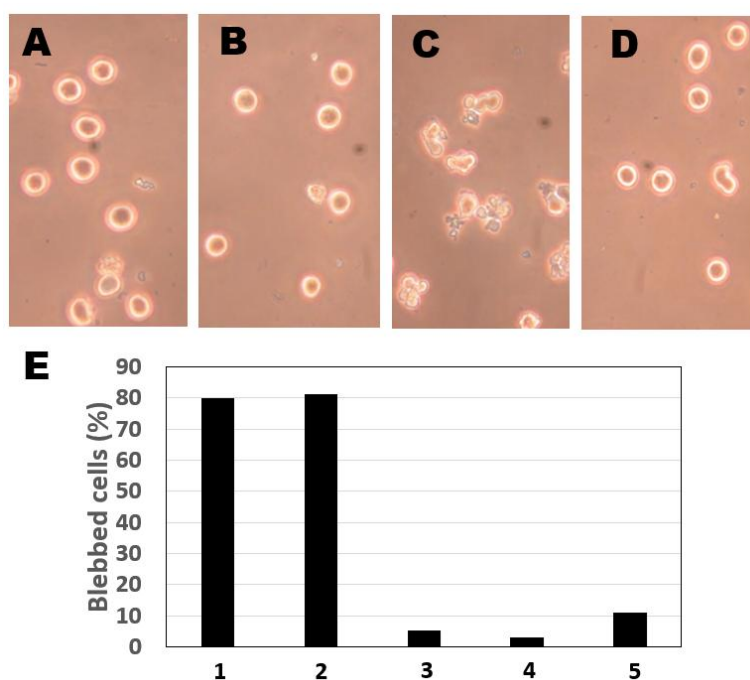
We have previously reported that hyperthermia induces apoptosis in prometaphase-arrested H-HeLa cells more rapidly when the cells have been arrested in mitosis for a longer time [27], suggesting that induction of apoptosis by hyperthermia depends on some factor that either accumulates or is depleted as prometaphase

arrest is prolonged. Fig. 7 shows that the same is true for apoptosis induced by treatment with ABT-263 at 42°C. This experiment used HeLa MKF-cdk1-as cells, in which Cdk1 is inhibited by the ATP analog 1NM-PP1 [34,35]. Monolayer cultures were synchronized at the G2/M boundary by treatment with 1NM-PP1 and then released into fresh medium containing nocodazole. After 1 hr, mitotic cells (mitotic index 93%) were harvested by shake-off. Some were incubated immediately with ABT-263 at 42°C, while others were kept an additional 4 hrs at 37°C before being treated with ABT-263 at 42°C. Induction of apoptosis (as judged by cell blebbing) proceeded at a significantly faster rate in the culture aliquot that had been arrested in mitosis longer.



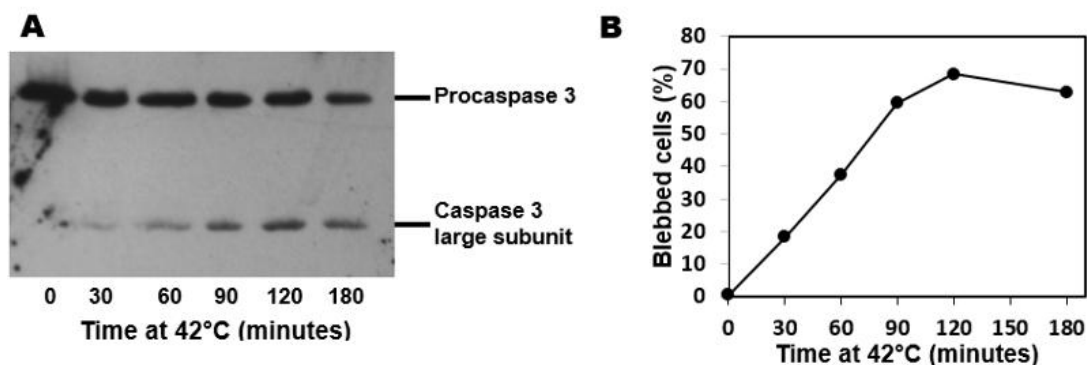
**Fig. 4. Apoptosis is induced in prometaphase-arrested HeLa S3 cells but not in interphase cells and requires both heat and ABT-263 treatment**

Aliquots of prometaphase-arrested (mitotic index 85%) and interphase (unsynchronized) HeLa S3 cell cultures were treated for 2 hrs at 42.0°C or 34.0°C either with (+) or without (-) 5 μM ABT-263



**Fig. 5. Induction of cell blebbing in prometaphase-arrested HeLa S3 cells (mitotic index 73%) by treatment with heat (42°C) and ABT-263 (5 μM) is blocked by the pan-caspase inhibitor zVAD-fmk (200 μM) or the caspase 3 inhibitor zDEVD-fmk (100 μM)**

(A) Cells at  $T = 0$ , before treatment; (B) Cells incubated for 60 min at 42°C with no drug treatment; (C) Cells treated with ABT-263 for 60 min at 42°C; (D) Cells treated with ABT-263 and zVAD-fmk for 60 min at 42°C. (E) Percentage of blebbed cells in culture aliquots incubated for 2 hr at 42°C with (1) 5 μM ABT-263; (2) 1 μM ABT-263; (3) 1 μM ABT-263 and 100 μM zDEVD-fmk; (4) 1 μM ABT-263 and 200 μM zVAD-fmk; and (5) no drug treatment (control)



**Fig. 6. Cleavage and activation of caspase 3 during treatment of prometaphase-arrested HeLa S3 cells (mitotic index 63%) with ABT-263 (5 μM) at 42°C**

At various times, samples were taken for microscopy and whole cell samples were prepared for SDS-polyacrylamide gel electrophoresis and western immunoblotting with an anti-caspase 3 antibody. (A) Appearance of cleaved caspase 3 as a function of time. (B) Percentage of blebbed cells as a function of time

#### 4. DISCUSSION

We have shown that treatment with the Bcl-2 inhibitor ABT-263 (Navitoclax) and mild hyperthermia (42°C) induces apoptosis in prometaphase-arrested HeLa S3 cells but not in

interphase cells. Similar results have been obtained with H-HeLa(WML) and HeLa MKF-cdk1-as. Occurrence of apoptosis is evidenced by morphological changes in the cells (blebbing of the plasma membrane), by the appearance of cleaved caspase 3, and by the fact that the

morphological changes are blocked by the caspase inhibitors zVAD-fmk and zDEVD-fmk. Far fewer apoptotic cells are observed when prometaphase-arrested cells are treated at 42°C in the absence of ABT-263, and ABT-263 does not induce apoptosis to a significant extent at 34°C.

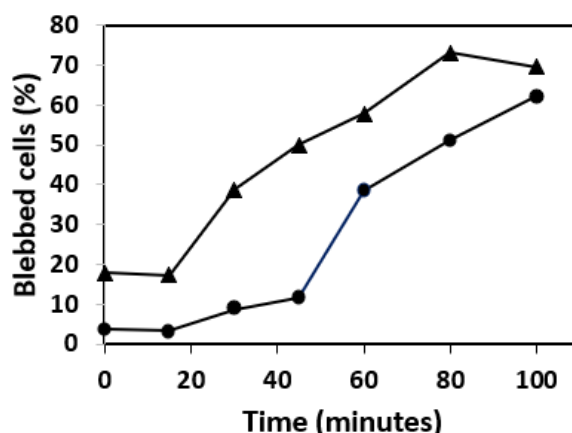
ABT-263 [41] is known as an inhibitor of several antiapoptotic proteins of the Bcl-2 family. It inhibits Bcl-2, Bcl-XL and Bcl-W with  $K_i \leq 1$  nM and inhibits Mcl-1 with  $K_i = 550$  nM and Bcl2A1 with  $K_i = 354$  nM [29]. It is likely that the effect we have observed is not due solely to inhibition of Bcl-2 but also depends on inhibition of one or more of the other proteins. In preliminary experiments we find that treatment of prometaphase-arrested HeLa S3 cells at 42°C with 5  $\mu$ M ABT-199 also induces apoptosis, but more slowly than comparable treatment with ABT-263 (unpublished data). ABT-199 is a more specific inhibitor of Bcl-2 than ABT-263 [43-45].

We previously showed [27] that prometaphase-arrested H-HeLa cells [26] rapidly undergo apoptosis when treated at 39-42°C, but prometaphase-arrested cells of other HeLa strains such as HeLa S3 do not [27]. Reconsideration of those results in light of the current work suggests that the different strains of HeLa we have examined may differ in their expression of various pro- or anti-apoptotic Bcl-2 family proteins. For example, H-HeLa may be deficient in Bcl-2 or a related protein. This

hypothesis should be testable. It is tempting to speculate that the unusual sensitivity of H-HeLa compared to other strains may somehow be related to its distinguishing characteristic, namely its susceptibility to infection by rhinoviruses and poliovirus [26,46].

However, down-regulation or deficiency of the Bcl-2 family proteins targeted by ABT-263 cannot be the only factor responsible for sensitivity to hyperthermia. There must be something else that distinguishes prometaphase-arrested from interphase HeLa cells. Otherwise, interphase HeLa S3 cells would also be susceptible to hyperthermia-induced apoptosis in the presence of ABT-263, but they clearly are not (cf. Figs. 1, 3 and 4).

Our aim is to determine why cells arrested in mitosis are more sensitive to hyperthermia than interphase cells. Several reasonable possibilities can be imagined, but one attractive hypothesis is that there may be a lower level of Mcl-1 in prometaphase-arrested cells. Mcl-1 is a short-lived antiapoptotic Bcl-1 family protein that is also liable to APC/C-dependent destruction during mitotic arrest [47-49]. Since transcription and protein synthesis are switched off during mitosis (e.g., [50,51]), the instability of Mcl-1 should lead to a decline in its concentration during prolonged mitotic arrest. Our observation that apoptosis is induced more readily when cells have been longer in prometaphase-arrest (Fig. 7 and [27]) is consistent with this hypothesis. The hypothesis



**Fig. 7. Apoptosis is induced more rapidly in cells that have been longer in mitotic arrest**  
*HeLa MKF-cdk1-as* cells were synchronized at the G2/M boundary by treatment for 20 hrs with 1NM-PP1 and then released into fresh medium containing nocodazole. After 1 hr, mitotic cells (mitotic index 93%) were harvested by shake-off. Aliquots of these mitotic cells were treated with 5  $\mu$ M ABT-263 at 42°C at either 1 hr (●—●) or 5 hr (▲—▲) after removal of 1NM-PP1. The percentage of cells with blebbed plasma membranes is shown as a function of the time at 42°C. Note that plasma membrane blebbing is induced more rapidly in the cells that had been in prometaphase-arrest 4 hr longer



also predicts that inhibition of Mcl-1 should magnify the effects of ABT-263 or ABT-199 and hyperthermia, and preliminary experiments indicate that this is the case. Treating prometaphase-arrested HeLa S3 at 42°C with the Mcl-1 inhibitor S63845 [52,53] as well as ABT-263 or ABT-199 leads to much more rapid blebbing than treatment with any of these inhibitors alone (unpublished work). It also leads to induction of apoptosis in interphase cells. Induction of apoptosis with a combination of ABT-199 and S63845 has also been reported [54]. Some of our preliminary work suggests that treatment with S63845 plus ABT-263 or ABT-199 can also induce apoptosis at lower temperatures, though more slowly than at 42°C. This raises the question, to what extent does hyperthermia have a specific effect in the induction of apoptosis and to what extent is it simply speeding it up?

The results with ABT-263 reported in this paper show that there exist conditions under which apoptosis is induced in prometaphase-arrested cells at 42°C but not at 34°C, suggesting that there may very well exist conditions under which the same is true of interphase cells. Identification of the relevant components and processes may make it possible to mimic, in interphase cells, the changes that sensitize metaphase-arrested cells to hyperthermia. This could make possible new treatments for cancer in which systemic drug treatment is combined with localized hyperthermia to help destroy a tumor.

## 5. CONCLUSION

The results presented here show that inhibition of Bcl-2 family antiapoptotic proteins with ABT-263 sensitizes prometaphase-arrested HeLa cells to apoptosis induced by mild hyperthermia. However, interphase cells are not affected by this treatment. It remains to be determined why prometaphase-arrested cells are more susceptible than those in interphase. If the features of mitotic-arrested cells that make them sensitive could be identified and mimicked in interphase cells by appropriate drug treatments, it may be possible to develop an effective new treatment for tumors that combines systemic chemotherapy with local hyperthermia.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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