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The BH3 Mimetic ABT-737 Induces Cancer Cell Senescence

Jin H. Song¹, Karthikeyan Kandasamy², Marina Zemskova³, Ying-Wei Lin⁴, and Andrew S. Kraft^{2,5}

¹Departments of Biochemistry and Molecular Biology, The Medical University of South Carolina, Charleston, South Carolina

²Department of Medicine, The Medical University of South Carolina, Charleston, South Carolina

³Department of Cell and Molecular Pharmacology, The Medical University of South Carolina, Charleston, South Carolina

⁴Department of Pediatrics, The Medical University of South Carolina, Charleston, South Carolina

⁵The Hollings Cancer Center, The Medical University of South Carolina, Charleston, South Carolina

Abstract

ABT-737, a small molecule cell-permeable Bcl-2 antagonist that acts by mimicking BH3 proteins, induces apoptotic cell death in multiple cancer types. However, when incubated with this agent many solid tumor cell lines do not undergo apoptosis. The current study reveals a novel mechanism whereby ABT-737 when added to apoptosis-resistant cancer cells has profound biologic effects. In PV-10 cells, a renal cell carcinoma that does not die after ABT-737 treatment, this agent induces a two-fold change in the transcription of nearly 430 genes. Many of these induced mRNA changes are in secreted proteins, IL-6, IL-8, and IL-11 and chemokines CXCL2 and CXCL5, or genes associated with an "inflammatory" phenotype. Strikingly, these gene changes are highly similar to those changes previously identified in cellular senescence. Brief exposure of apoptosis-resistant renal, lung and prostate cancer cell lines to ABT-737, although not capable of inducing cell death, causes the induction of senescence-associated β -galactosidase and inhibition of cell growth consistent with the induction of cellular senescence. Evidence indicates that the induction of senescence occurs as a result of reactive oxygen species elevation followed by low-level activation of the caspase cascade, insufficient to induce apoptosis, but sufficient to lead to minor DNA damage and increases in p53, p21, IL-6 and 8 proteins. By overexpression of a dominant-negative p53 protein, we show that ABT-737-induced cellular senescence is p53-dependent. Thus, in multiple cancer types in which ABT-737 is incapable of causing cell death, ABT-737 may have additional cellular activities that make its use as an anticancer agent highly attractive.

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Corresponding Authors: Jin H. Song, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425. Phone: 843-792-9366; Fax: 843-792-3200. songjin@musc.edu; or Andrew S. Kraft, Department of Medicine, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425. Phone: 843-792-8284; Fax: 843-792-3200. kraft@musc.edu.

Current address for Karthikeyan Kandasamy: Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669.

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No potential conflicts of interests were disclosed.

Introduction

Aberrant expression of the antiapoptotic Bcl-2 family of proteins in human cancer is correlated with poor outcomes after standard chemotherapy. One approach to overcoming this blockade is the development of compounds such as ABT-737, a cell permeable small molecule Bcl-2 family antagonist that is capable of binding to Bcl-2, Bcl-x_L, and Bcl-w, but does not block the activity of Mcl-1, Bfl-1/A1 and Bcl-B (1). This agent functions by displacing BH3 proteins, such as Bim, from these proteins activating Bax and Bak to induce apoptotic cell death (2) and has the ability to kill leukemia and lymphoma (1), multiple myeloma (3), glioblastoma (4) and small cell lung cancer cell lines (5). On the basis of these results an orally active form of this compound, ABT-263, has entered clinical trials (6, 7).

Emerging results show that ABT-737, especially when used to treat solid tumors including lung, prostate, and renal cancers (1, 8), can be ineffective at inducing apoptosis. Resistance to ABT-737 is in part mediated by the elevated expression of Mcl-1 or Bfl-1/A1, which are not capable of binding this compound (9, 10). Carcinomas exhibit elevated levels of endogenous Mcl-1 and are resistant to killing by this agent (8, 11). Alternatively, we have suggested (8) that ABT-737, although not inducing cell death in tumor cells, could instead be an important anticancer agent functioning by regulating gene transcription sensitizing resistant tumor types to alternative therapies. For example, we have shown that application of ABT-737 transcriptionally induces increases in the level of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5 (8) enhancing cell killing by the addition of the proapoptotic protein TRAIL. Likewise, ABT-737 resistance in glioma and pancreatic cells expressing high levels of Mcl-1 was completely reversed by cotreatment of TRAIL (4, 12).

To investigate in more depth the extent of transcriptional changes induced by ABT-737 in tumor cells resistant to apoptosis, we have treated the renal carcinoma cell line PV-10 and examined changes in mRNA levels by microarray analysis. Gene chip analysis reveals that ABT-737 treatment regulates more than 400 genes many of which are cytokines and chemokines. Interestingly, we find that these gene changes have been previously identified (13–15) as markers of cellular senescence. Senescence is a complex programmed cellular response to multiple stimuli, including DNA damage, oncogene expression, or telomere shortening associated with aging, which is characterized by irreversible growth arrest accompanied by distinct morphologic changes such as an increased number of enlarged and flattened cells (16). On the basis of this initial result, we have further examined the biochemical mechanism regulating the ability of ABT-737 to inhibit tumor cell growth and induce the senescent phenotype.

Materials and Methods

Cell culture and reagents

Human cancer cell lines, including 22Rv1, DU145, A549, WI-38, purchased from the ATCC (authenticated by short tandem repeats single nucleotide polymorphism, and fingerprint analyses), and PV-10 cells (8) provided by Drs. R. Gemmill and H. Drabkin (Medical University of South Carolina, Charleston, SC), were used for senescence studies. Cell lines were cultured according to the supplier's instructions, and maintained at low passage number for less than 6 months. ABT-737 (A-779024.0) and its inactive enantiomer (A-793844.0) were a gift of Abbott Laboratories. Both compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 20 mmol/L and aliquots were stored at –80°C.

Viruses and infection

Human GIPZ lentiviruses encoding short hairpin microRNAs against *ATM* (V2LHS_89368) and *CAP3* (V2THS_15048 plus V2THS_15049), and a nonsilencing control were purchased from Open Biosystems. Arrest-In Lentiviral expression system (Open Biosystems) was used to establish PV-10 and 22Rv1 cell lines harboring small hairpin RNAs (shRNA). The following retroviral vectors were used in these experiments: pBabePuro-p53 (Addgene), expressing DN-p53 (17), and pLNCX-GFP (Clontech Laboratories Inc.) as previously described (18).

Cell viability assays

Cells were seeded in 96-well plates or culture dishes and treated with ABT-737. Cell growth was determined by a 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich) using a microplate reader. The percentage of viable cells was evaluated by trypan blue exclusion assay as previously described (8). For clonogenic assays, cells were plated at 1,000 cells per well and cultured in medium with 0, 1, 5, 10 $\mu\text{mol/L}$ of ABT-737 for 2 to 3 weeks. The cells were stained with crystal violet (19).

BrdU assay

Cell proliferation assays was performed using a bromodeoxyuridine (BrdU) cell proliferation assay kit (CalBiochem). After 24 hours of BrdU labeling, the relative and background signal was assessed by using a fluorescent plate reader (Synergy 2, BioTek).

IL-6 and IL-8 ELISA, and SA- β -gal assay

Cells (1×10^5) were plated and after 24 hours, fresh medium was added. Conditioned medium was collected following treatment and used at a 2:1 dilution in the human interleukin-6 (IL-6) or IL-8 ELISA Kit (R&D Systems) according to the manufacturer's instructions. SA- β -gal activity was determined using a SA- β -gal staining kit from Cell Signaling Technology according to the manufacturer's instruction. Senescent cells were identified as blue-stained cells by standard light microscopy, and a total of 500 cells were counted in 3 random fields on a slide to determine the percentage of SA- β -gal-positive cells.

Western blot analysis

Western blot analysis was done as described previously (8) and is described in detail in the Supplementary Material and Methods.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) is described previously (20) and in detail in the Supplementary material.

Real-time PCR, microarray, and luciferase assays

Real-time PCR, primers and microarray gene expression profiling are performed as described in detail in the Supplementary Experimental Procedures. Dual luciferase assay was performed (8) and is described in detail in the Supplementary materials.

Results

ABT-737 induces the transcriptional activation of IL-6 and IL-8

We have identified a group of cancer cell lines (8) that are resistant to the proapoptotic activity of ABT-737. To examine changes in gene transcription after ABT-737 treatment of PV-10 cells, which are a kidney cancer cell line resistant to apoptosis induced by this agent,

we carried out microarray analysis using an Affymetrix human gene chip (U133 Plus 2.0 Array) with mRNA from cells either treated with vehicle or ABT-737 for 24 hours. Our analysis of these results revealed that more than 430 genes were significantly changed (>2-fold, $P < 0.05$) either up or down after exposure to ABT-737 (Fig. 1A). Genes that are regulated more than 4-fold by ABT-737 are shown in (Supplementary Table S1). *IL-6*, *IL-8*, and *IL-11* and chemokines *CXCL2* and *CXCL5* and other secreted cytokines/chemokines are induced more than 2-fold (Fig. 1B). Quantitative real-time PCR (qT-PCR) analysis of *IL-6* and *IL-8* showed that ABT-737, but not its inactive enantiomer, was able to induce changes in mRNA levels confirming the microarray analysis (Fig. 1C). Significant increases in other mRNA transcripts including *IL-11*, *CXCL2*, *CXCL5*, *IRF1*, and *GADD45A* were also confirmed by qT-PCR analysis (Supplementary Fig. S1). To further validate gene changes shown in the microarray analysis, we also included genes that are downregulated by ABT-737 treatment. QT-PCR analysis confirmed that ABT-737 inhibited expression of inhibitor of differentiation 2 (*ID2*) and *ID3* transcripts (Supplementary Fig. S1). To determine whether the change in *IL-6* and *IL-8* mRNA levels is mirrored with a change in the protein produced, we performed an ELISA assays. Secretion of *IL-6* and *IL-8* protein was both increased in PV-10 cells in response to ABT-737 treatment (Fig. 1D). To examine whether these gene changes were seen in other cell lines after ABT-737 treatment, we studied the effect of ABT-737 on 6 additional cancer lines, 4 renal cancer cell lines, KRC/Y, KV-6, CAKI, and 786-0, and 2 lung cancer cell lines, A549 and HOP-62. In all of these cell lines with only minor exceptions, ABT-737 was able to induce *IL-6* and *IL-8* and downregulate *ID2* and *ID3* transcripts (Supplementary Fig. S2). Pathway analysis shows marked changes in genes associated with protein binding, signal transduction, apoptosis in addition to changes in cytokine activity, and inflammatory response mediators.

Gene microarray analysis revealed that ABT-737 induced a significant increases in the transcription factor CCAAT/enhancer-binding protein beta (C/EBP β ; Fig. 1B). Consistent with these results, qT-PCR and Western blot analysis showed significant induction of C/EBP β mRNA and protein levels in PV-10 cells treated with ABT-737 (Fig. 2A and B). Because the *IL-6* promoter contains both C/EBP β and NF- κ B binding sites, we examined the possibility that activation of these 2 transcription factors played an important role in regulating *IL-6* mRNA levels. Previously, we had reported that NF- κ B promoter activity is increased by ABT-737 (8) and EMSA show that ABT-737 treatment of PV-10 cells activates NF- κ B activity (Fig. 2C). We find that ABT-737 treatment, but not the inactive enantiomer, of PV-10 cells was able to activate both the *IL-6* and *IL-8* promoters (Supplementary Fig. S3). Using a plasmid in which the *IL-6* promoter was cloned in front of a luciferase reporter, mutation of either the C/EBP β or NF- κ B sites (Fig. 2D), blocked the transcriptional activation of this gene by ABT-737. These DNA microarray results show that in a cell line that does not undergo apoptosis after treatment ABT-737 is capable of inducing changes in gene transcription.

Irreversible cell cycle arrest and senescence is induced by ABT-737 treatment

The induction of *IL-6*, *IL-8*, *CXCL2*, and *CXCL5* and other secreted proteins has been identified (13, 15) as associated with the induction of cellular senescence. Senescent cells secrete multiple factors that alter tissue microenvironments, including the inflammatory cytokines such as *IL-6* and *IL-8* (13, 15, 21). These cytokines and chemokines function to reinforce the senescent growth arrest through autocrine and paracrine mechanisms (21, 22). The transcription factor C/EBP β cooperates with *IL-6* to amplify the activation of inflammatory mediators, including *IL-8* (15).

To test senescence induction, we first examined the effect of ABT-737 on PV-10 cell growth. Long-term exposure of PV-10 cells to ABT-737 induced significant growth inhibition and a G₁/S cell cycle blockade (Fig. 3A; Supplementary Fig. S4). Direct detection

of apoptosis with Annexin V staining showed that apoptotic cells were not significantly increased (Supplementary Fig. S4). However, a dose-dependent inhibition of colony formation and an inhibition of BrdU incorporation was seen after the addition of ABT-737 to both PV-10 and 22Rv1 cells (Fig. 3B and C). To test the possibility that cell growth arrest in response to ABT-737 was caused by the induction of cellular senescence, 5 human carcinoma cell lines including PV-10 cells were exposed to ABT-737 followed by staining with SA- β -gal, a marker of senescent cells. PV-10, A549, and 22Rv1 cells stained positively for this senescent marker whereas WI-38 cells, normal human fibroblasts, and DU145 human prostate cancer cells showed no response (Fig. 3D and E). Cells that were positive for SA- β -gal showed a flattened and enlarged morphology that was consistent with senescence. Although the WI-38 cell line was resistant to ABT-737 induced senescence, γ -irradiation (IR) caused senescence-like morphologic changes in WI-38 human fibroblast cells, suggesting that ABT-737 did not induce senescence in all cell lines.

DNA damage is induced by ABT-737

Overwhelming DNA damage leads to apoptosis, but lower levels of damage have been suggested to be as a causal factor in the induction of both cellular senescence and growth arrest (23). To investigate whether ABT-737 induced lower levels of DNA damage, we investigated the ability of this agent to induce DNA double-strand breaks (DSB), followed by the stimulation of ataxia telangiectasia mutated (ATM) kinase-dependent phosphorylation of the histone H2A variant H2AX, γ -H2AX (7). Western blot analysis revealed a time and dose-dependent induction of both ATM phosphorylation and γ -H2AX in PV-10 cells, 22Rv1, and A549 cells (Fig. 4A and B). Similar results were revealed by immunofluorescent staining of PV-10 cells showing that ABT-737 and DNA damaging agents IR or doxorubicin (DXR) caused the induction of γ -H2AX (Supplementary Fig. S5). Recently, chromatin structures associated with modified histones, such as dimethyl-histone H3 at Lys9 (H3K9m), were identified as a critical feature of cellular senescence. During senescence H3K9m increases and contributes to the transcriptional repression of growth promoting genes found in senescence (24, 25). Using an anti-H3K9m antibody to stain treated and untreated cells, immunofluorescence microscopy shows that ABT-737, as well as DXR and IR, induced this histone modification (Supplementary Fig. S5).

To test the role of the ATM protein kinase as a mediator of the induction of senescence by ABT-737, we infected PV-10 and 22Rv1 cells with lentiviruses encoding short hairpin microRNA targeting ATM (shRNAmir-ATM). The initiation and maintenance of senescence-associated cytokine secretion requires the DNA damage response proteins, including ATM kinase, nijmegen breakage syndrome 1, and check point kinase 2 (26). In a pool of PV-10 cells ATM levels were decreased by shRNA, we found that a marked reduction in ATM levels prevented ABT-737 from inducing γ -H2AX (Fig. 4C). Moreover, ABT-737 treatment of cells with lower levels of ATM protein kinase caused a markedly reduced level of SA- β -Gal and senescence-like morphologic changes (Fig. 4D). To examine whether the induction of DSBs followed by ATM activation was the driving force behind the ABT-737-mediated senescence induction of IL-6 and IL-8, we measured levels of *IL-6* and *IL-8* mRNA in PV-10-shRNAmir-ATM and PV-10-shRNAmir-control cells. QT-PCR analysis showed that ATM knockdown prevented the ABT-737-mediated induction of *IL-6* and *IL-8* mRNA (Supplementary Fig. S6). Similar results were observed in 22Rv1 prostate cancer cells depleted of ATM. These findings support the concept that activation of ATM by ABT-737-induced DNA damage signals the induction of senescence leading to an increase in *IL-6* and *IL-8* mRNA.

Contribution of caspase-3 cleavage to DNA damage response and senescence

To examine whether the DNA damage response caused by ABT-737 occurs as a result of activation of the caspase cascade or was secondary to an off-target direct effect of this drug, we treated cells with z-VAD-FMK, a pan-caspase inhibitor. As a positive control, in these experiments we added TRAIL to ABT-737, a combination we have previously showed is highly synergistic and induces caspase activation and apoptosis (8). When PV-10 cells were treated with ABT-737, caspase-3 (p32 precursor) is processed into 1 inactive (p24) and 2 active (p20 and p17) cleavage fragments. The cleaved subunit of caspase-3 generated in response to ABT-737 is insufficient to further process poly(ADP-ribose) polymerase (PARP), a substrate commonly cleaved during apoptosis. However, the γ -H2AX induction by ABT-737 correlated with caspase-3 cleavage, suggesting the possibility that caspase-3 cleavage induced by ABT-737 might be the cause of the DNA damage response (Fig. 5A). Because z-VAD-Fmk is not a specific caspase-3 inhibitor, we depleted caspase-3 levels in PV-10 and 22Rv1 cells with lentiviral infection of shRNA_{mir} against caspase-3, and examined the ability of ABT-737 to induce senescence. Western blot analysis confirmed in both PV-10 and 22Rv1 cells that partial knockdown of caspase-3 expression inhibited the ABT-737 induction of γ -H2AX (Fig. 5B). To initiate DSBs, caspase-3 results in the activation of caspase-activated DNase (CAD) by cleaving its inhibitor ICAD (27). We show that ABT-737 treatment of PV-10 cells induced ICAD cleavage, which was absent in caspase-3 knockdown cells (Fig. 5C). Exposure of ABT-737 to cells with decreased caspase-3 expression induced a reduced percentage of SA- β -gal-positive cells compared with those cells treated with shRNA_{mir}-control (Fig. 5D). QT-PCR analysis also showed that caspase-3 knockdown prevented the ABT-737-mediated induction of *IL-6* and *IL-8* mRNA (Fig. 5E). These results support the hypothesis that ABT-737 produced insufficient levels of cleaved caspase-3 to drive apoptosis, but sufficient levels to cause the DNA damage-mediated induction of senescence and induction *IL-6* and *IL-8* (13, 15, 26).

Because reactive oxygen species (ROS) is known to be an inducer of senescence, we evaluated whether treatment of PV-10 and 22Rv1 cells with ABT-737 might increase oxidative stress. ROS levels, as assessed by dichlorofluorescein, after ABT-737 treatment of both PV-10 and 22Rv1 cells were increased (Fig. 6A), and *N*-acetyl cysteine (NAC), a precursor of intracellular glutathione, prevented this ROS increase. NAC also partially blocked ABT-737-mediated cleavage of caspase-3 and ICAD suggesting that activation of caspase-3 and DNA damage is in part due to the elevation of ROS (Fig. 6B). Consistent with the role of DNA damage in the induction of senescence, NAC treatment also reduced percentages of SA- β -gal-positive cells (Fig. 6C).

Requirement for the activation of p53 and induction of p21 in ABT-737-driven senescence

Both DNA damage and the induction of senescence are associated with the activation of p53 and the transcription of genes, such as p21 (28). Increases in cell cycle inhibitor p21 and p16 gene transcription are thought to play a critical role in the mechanism by which cells undergo senescence (29, 30). Both PV-10 and 22Rv1 cells when treated with ABT-737 show increased levels of both wild-type p53 and p21 protein levels (Fig. 7A), but no change in p16 mRNA levels (Supplementary Fig. S1A). As predicted from the changes in p53, the upregulation of p21 protein levels occurred at the transcriptional level and the enantiomer had no activity in regulating p21 (Fig. 7B). To examine whether ABT-737-induced increases in p21 levels could account for the growth arrest seen after treatment, we measured the effect of ABT-737 on the activity of the Cdk2 protein, a kinase that plays an important role both in controlling the G₁/S transition, and has a clear impact on the induction of oncogene-induced senescence (31). The Cdk2 kinase was immunoprecipitated from treated cells and incubated with histone H1 as a substrate. We find that ABT-737 treatment inhibits

Cdk2 activity in a dose-dependent fashion in both PV-10 and 22Rv1 cells (Supplementary Fig. S7).

To examine whether the p53 pathway is essential for the induction of senescence, we generated a pool of 22Rv1 cells expressing dominant-negative p53 (DN p53; rev. 17). Western blot analysis confirmed that after ABT-737 treatment overexpression of DN p53 caused a substantial reduction in p21 (Fig. 7C) and inhibited the ability of ABT-737 to induce SA- β -Gal (Fig. 7D). As a result of DN p53 expression, ABT-737 induction of increases in the *IL-6* and *IL-8* mRNA were also significantly reduced (Fig. 7E). To show whether changes in IL-6 and IL-8 are necessary for ABT-737 induction of senescence, C/EBP β protein was ablated by siRNA-treatment (Supplementary Fig. S8). QT-PCR analysis of these cells with C/EBP β knockdown showed that the ABT-737-mediated induction of *IL-6* and *IL-8* mRNA levels was significantly reduced (Supplementary Fig. S8). Similarly, senescence as measured by SA- β -gal staining induced by ABT-737 was also reduced by decreasing C/EBP β levels (Supplementary Fig. S8). Together, these results indicate a model whereby ABT-737 induces a signaling cascade including changes in ROS/Caspase-3/DDR/p53/p21/IL-6/IL-8 that leads to cancer cell senescence (Fig. 7F).

Discussion

We show that the addition of ABT-737 to cancer cell lines that do not undergo apoptosis (8) inhibits cancer cell proliferation associated with the induction of the senescent phenotype. The induction of senescence in both normal and cancerous cell types occurs with marked changes in gene transcription and protein secretion (13) including significant secretion of cytokines and chemokines normally associated with the inflammatory response. Likewise, we have found that the application of ABT-737 to PV-10 kidney cancer cells regulated the transcription of more than 400 genes, many of which are associated with an "inflammatory" phenotype (15, 21). We have identified marked increases in IL-6 and IL-8 driven by changes in the levels of the C/EBP β transcription factor, mimicking defined events delineated in oncogene-induced and other types of senescence (13, 32). The senescent phenotype was further shown by the induction of SA- β -gal staining, a flattened morphology, a marked decrease in BrdU incorporation, and a G₁/S cell cycle blockade. Together these results show that in a solid tumor cell lines cells that do not undergo apoptosis after ABT-737 treatment, this compound is capable of inducing senescence.

We propose that ABT-737, although not inducing apoptotic levels of DNA damage, is capable of inducing low levels of DNA damage as measured by increases in γ -H2AX and ATM phosphorylation. Both the pan-caspase inhibitor z-VAD-FMK and expression of a shRNA specific to caspase-3 blocked the ability of ABT-737 to induce γ -H2AX, showing that activation of this caspase by ABT-737 was essential to the induction of DNA damage. Activation of caspase-3 has been implicated in specific gene regulation, for example, in the repression of CD28 expression in aging lymphocytes (33). More recently, activation of both CAD and caspase-3 have been shown to be necessary for myoblast differentiation and to induce *p21* mRNA transcription (34). Caspase activation was sufficient to induce biologic changes, but insufficient to induce apoptosis. Activation of ROS production has been associated both with the regulation of caspase-3 activity and the induction of senescence (35, 36). As suggested by the ability of NAC to partially block both caspase-3 activation and senescence, the addition of ABT-737 to these tumor cells ROS plays a role in low level caspase-3 activation. Similarly, it has been reported that peroxynitrite leads to senescence of red blood cells through caspase-3 activation (37). Very low doses of γ -radiation induce only transient DNA damage that is quickly repaired. Higher doses of radiation are needed for prolonged DNA-damage that then leads to the induction of senescence-associated markers (26), and still higher doses are needed to induce cell death. Finally, the amount and extent of

DNA damage has been linked to the ability of cells to increase the secretion of the immune mediator cytokines/chemokines associated with senescence (38), similar to what is seen here.

Increases in p21 is an essential control point in senescence (30) not only because modulation of this protein blocks cyclin-dependent protein kinases, but also because p21 regulates the transcriptional activation of multiple genes necessary for the induction of the senescent phenotype (39). In multiple tumor types, activation of the p53 (40) has been shown to be essential for the induction of senescence. Using overexpression of dominant-negative p53, we show that the p53 pathway is also necessary for ABT-737 senescence in the 22Rv1 prostate tumor cells. Other DNA damaging agents require p53 as an essential mediator of senescence (41). On the basis of our experiments, we hypothesize that treatment of p53-positive solid tumor patients with ABT-737, or its oral counterpart ABT-263, may yield significant stable disease based on the induction of cellular senescence. Future human clinical trials will be needed to evaluate this important possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Gene transcription changes after ABT-737 treatment. A, microarray analysis of gene expression induced by ABT-737. PV-10 cells were treated in triplicate with DMSO or 10 $\mu\text{mol/L}$ ABT-737 for 24 hours and gene microarray changes documented. B, gene changes associated with senescence. C, qT-PCR analysis of IL-6 and IL-8 transcripts. PV-10 and 22Rv1 cells were treated in triplicate with DMSO, ABT-737 (10 $\mu\text{mol/L}$) or enantiomer (En., 10 $\mu\text{mol/L}$) for 24 hours (mean $\pm$ SD, $n = 3$). D, the secretion of IL-6 and IL-8 in PV-10 cells treated with DMSO, ABT-737 or enantiomer for 24 hours was determined by ELISA ($n = 6$, mean $\pm$ SD).

Figure 2.

Transcriptional regulation of IL-6 increased by ABT-737 is mediated by transcription factors C/EBPβ and NF-κB. A, qT-PCR analysis of C/EBPβ mRNA expression. PV-10 cells were treated with DMSO or 10 μmol/L ABT-737 (ABT) for 24 hours (triplicate, ±SD). B, C/EBPβ protein expression is determined by Western blotting. C, EMSA on nuclear lysates from PV-10 cells with an NF-κB radiolabeled probe. Competition with wild type (100-fold excess) was used to show the specificity of this assay. Nuclear extracts from PV-10 cells treated with TNFα (3 nmol/L) for 30 minutes were used as positive control. D, luciferase reporter assay of IL-6. PV-10 cells were cotransfected with a luciferase construct, a wild-type (Wt-luc), NF-κB (–ΔNF-κB-luc), or C/EBPβ (–ΔC/EBPβ-luc) deletion mutant, and 0.01 μg of pEF-*Renilla*-luc. IL-6 luciferase activity (mean ± SD, *n* = 3) was determined by dual luciferase assay as described in Materials and Methods. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 3.

ABT-737 induces senescent-like growth arrest in human cancer cells. A, cell growth curves were determined by MTT assay. PV-10 cells were treated with DMSO or ABT-737 (ABT; 10 $\mu\text{mol/L}$) for 24 hours only, the cell washed and then maintained in fresh growth media for up to 6 days (triplicate, $\pm\text{SD}$). B, BrdU incorporation. The percentage of BrdU-positive cells was determined 5 days after ABT-737 exposure. C, clonogenic assay. Cells were treated with the indicated doses of ABT-737 for 5 days. The colony formation was visualized by crystal violet staining. The bar graph indicates the percentage inhibition of colony formation from control (DMSO). D and E, SA- β -gal staining. At 5 days after exposure to ABT-737, SA- β -gal-stained cells were visualized under phase contrast microscopy (D). The bar graph indicates percentage of SA- β -gal-positive cells (E) in each cell type.

Figure 4.

DNA damage response induced by ABT-737 for induction of senescence. A, induction of γ -H2AX and p-ATM by ABT-737. Cells were treated with 10 μ mol/L ABT-737 for 24 hours and then maintained in fresh media for 1 or 2 more days prior to Western blot analysis. B, Western blot analysis of dose-dependent induction of γ -H2AX expression. C, Western blot analysis of ATM and γ -H2AX expression levels. ATM expression in PV-10 cells was decreased by lentiviral infection with short hairpin microRNA (shRNAmir) targeting the ATM (shRNAmir-ATM). A nonsilencing control (shRNAmir-control) was also employed. Cells were treated with DMSO (–) or 10 μ mol/L ABT-737 (+) for 24 hours. D, SA- β -gal activity in PV-10 cells infected with nonsilencing shRNAmir or ATM shRNAmir. These cells were exposed to DMSO or ABT-737 for 24 hours, washed with PBS, and then maintained in fresh growth media for 5 days. After staining with β -gal, the percentage of SA- β -gal-positive cells was evaluated under phase contrast microscopy (left). Phase contrast microscopy of SA- β -gal-positive cells is shown (right).

Figure 5.

Contribution of caspase-3 cleavages to the ABT-737-induced senescence. A, Western blot analysis of caspase cleavage. PV-10 cells were treated with 10 μ mol/L of ABT-737 with or without 40 μ mol/L of z-VAD-FMK for 24 hours. Whole-cell lysates were subjected to Western blot analysis and the cleavage fragments of caspases identified by arrows. B, Western blot detection of γ -H2AX expression after caspase-3 (casp-3) knockdown. After 24 hours treatment, extracts were Western blotted with multiple antibodies. C, Western blot analysis of ICAD cleavage. The cleavage fragments of ICAD are identified by arrows. D, SA- β -gal staining was done at day 5 after exposure of ABT-737 or DMSO. E, qT-PCR analysis of *IL-6* and *IL-8* mRNA in cells depleted without (shRNAmir-Control) or with

(shRNAmir-Casp-3) casp-3 expression. Cells were exposed to ABT-737 for 24 hours and then qT-PCR analysis (the mean $\pm$ SD of triplicate experiments of these values) was done. The IL-6 and IL-8 mRNA levels were normalized to GAPDH. DFF45, DNA fragmentation factor 45.

Figure 6.

ABT-737 treatment increases the generation of ROS. A, ROS measurement. ROS levels were assessed by dichlorofluorescein (DCF) measurement after ABT-737 treatment for 24 hours. The data represent the mean $\pm$ SD of 3 independent experiments. B, Western blot analysis of caspase-3 and ICAD cleavage. Cells were pretreated with *N*-acetyl cysteine (NAC; 5 mmol/L) for 2 hours followed by DMSO or ABT-737 treatment for 24 hours. C, SA- β -gal activity. Cells were exposed to DMSO or ABT-737 for 24 hours in presence or absence of NAC, washed with PBS, and then maintained in fresh growth media for 5 days. After staining with β -gal, the percentage of SA- β -gal-positive cells was evaluated under

phase contrast microscopy (triplicate experiments, the mean $\pm$ SD of measured values is shown).

Figure 7.

ABT-737 induces cell cycle arrest through activation of p53-p21. A, Western blot analysis of p21 and p53 levels. Cells were treated with DMSO (–) or ABT-737 (10 μmol/L) for 24 hours and cellular extracts subjected to Western blotting. B, qT-PCR analysis of p21 transcripts. Cells were treated with DMSO, ABT-737, or enantiomer (En.) for 24 hours. The p21 mRNA levels were normalized to GAPDH. C, inactivation of p53 blocks ABT-737–mediated senescence. The p53 function in 22Rv1 cells was inactivated by retroviral infection with pBabe-hygro dominant-negative p53 (DN p53) and selected with hygromycin (150 μg/mL) for 14 days. These cells were treated with DMSO or 10 μmol/L ABT-737 (24 hours pretreatment) prior to Western blot analysis for p21 and p53. D, SA-β-gal activity.

PV-10 cells in the top were treated for 24 hours and senescent cells evaluated 5 days later. The percentage of SA- β -gal-positive cells is given in the right and the morphologic features are shown in the left. PV-10 cells were treated with 500 μ mol/L hydrogen peroxide (H_2O_2 ; for 2 hours pretreatment) and evaluated for SA- β -gal activity at day 5. E, qT-PCR analysis of *IL-6* and *IL-8* mRNA in 22Rv1 cells expressing dominant-negative p53. 22Rv1 cells expressing empty vector (EV) or pBabe-hygro dominant-negative (DD) p53 (DN p53) were treated with DMSO (–) or 10 μ mol/L ABT-737 (+) for 24 hours. F, representative schematic diagram summarizing the signaling pathway for cancer cell senescence induced by ABT-737.