Circulating Biomarkers of Cell Death After Treatment with the BH-3 Mimetic ABT-737 in a Preclinical Model of Small-Cell Lung Cancer

Dimitra Micha,[1] Jeff Cummings,[1] Alex Shoemaker,[2] Steven Elmore,[2] Kelly Foster,[2] Martin Greaves,[1] Tim Ward,[1] Saul Rosenberg,[2] Caroline Dive,[1] and Kathryn Simpson[1]

Abstract

Purpose: This study evaluated epithelial cell death ELISAs that measure circulating cytokeratin 18 in mice bearing small-cell lung cancer xenografts treated with a proapoptotic dose of the BH-3 mimetic ABT-737.

Experimental Design: H146 tumor–bearing and non–H146 tumor-bearing severe combined immunodeficient (SCID)/*bg* mice were treated with ABT-737 or vehicle control. Plasma collected before and 2 to 360 hours after treatment was analyzed by M30 (caspase-cleaved cytokeratin 18) and M65 (intact and cleaved cytokeratin 18) ELISA. In parallel, tumors were interrogated for cleaved caspase-3 and cleaved cytokeratin 18 as biomarkers of apoptosis.

Results: ABT-737–treated tumors regressed by 48 hours ($P < 0.01$) compared with controls, correlating with increased cleaved cytokeratin 18 ($P < 0.01$; 6 and 24 hours) and increased intact cytokeratin 18 ($P < 0.01$; 24 hours). Cleaved cytokeratin 18 levels decreased below baseline between 72 and 360 hours for ABT-737–treated and control mice whereas intact cytokeratin 18 decreased below the level of detection at 8 and 15 days in ABT-737–treated mice only. Apoptosis in tumors reflected changes in circulating cytokeratin 18 (cleaved caspase-3, $P < 0.05$ at 2 hours and $P < 0.001$ at 6, 12, and 24 hours; caspase-cleaved cytokeratin 18, $P < 0.05$ at 15 days, for drug treated versus controls).

Conclusions: ABT-737 caused tumor regression by apoptosis in H146 xenografts that mapped to a drug-specific, early increase in circulating cleaved cytokeratin 18 that subsequently declined. Circulating, intact cytokeratin 18 levels correlated with tumor burden. Cleaved caspase-3 and caspase-cleaved cytokeratin 18 in tumor correlated with treatment ($P < 0.05$, 2 hours; $P < 0.001$, 6, 12, and 24 hours; cleaved caspase-3, $P < 0.05$, 15 days; caspase-cleaved cytokeratin 18), indicating that events in plasma were tumor derived. These circulating biomarker data will be translated to clinical trials wherein serial tumor biopsies are rarely obtained.

The arrival of molecularly targeted agents for the treatment of cancer brings added impetus to pharmacodynamic biomarker qualification and the need for biomarker-enhanced clinical trials wherein the proof of mechanism (drug hits target) and proof of concept (appropriate tumor response is stimulated) are sought (1). Drugs that target components of apoptotic pathway(s) are currently in preclinical development and entering early clinical trials (2). Validated proof-of-concept biomarkers that report apoptotic cell deaths in tumor or, as is often the case when tumor is not available, in surrogates such as blood are therefore urgently required (3–5). With this in mind, this study evaluates circulating biomarkers of epithelial cell death in a responsive preclinical tumor model before and after treatment with ABT-737, a proapoptotic Bcl-2 family–targeted novel agent (6).

The antiapoptotic Bcl-2 family of proteins are attractive drug targets because they are frequently overexpressed in many human cancers and can mediate drug resistance (7). Targeting these molecules requires the specific interruption of protein-protein interactions between proapoptotic and antiapoptotic family members that involve their BH-3 domains (8). The highly potent BH-3 mimetic, ABT-737, is the leading member of a new class of small-molecule drugs that is approaching or entering early clinical trials for cancer treatment (6). ABT-737 binds with nanomolar affinity to the BH-3 binding groove of antiapoptotic proteins Bcl-2, Bcl-x_L, and Bcl-w, but not to those of Mcl-1 and A1 (6, 9–12). Upon binding, ABT-737 prevents these antiapoptotic proteins from sequestering proapoptotic family members to trigger apoptosis via the intrinsic mitochondrial pathway (6). Preclinical studies have shown that ABT-737 sensitizes many cancer cell types to conventional therapies *in vitro* (6, 11, 13–19), and it

Authors' Affiliations: [1]Clinical and Experimental Pharmacology Group, Paterson Institute for Cancer Research, University of Manchester, Manchester, United Kingdom and [2]Abbott Laboratories, Abbott Park, Illinois

Received 1/15/08; revised 5/13/08; accepted 5/13/08.

Grant support: Cancer Research UK grant C147 (C. Dive) and Cancer Research UK studentship (D. Micha).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Caroline Dive or Kathryn Simpson, Paterson Institute for Cancer Research, Wilmslow Road, Manchester M20 4BX, United Kingdom. Phone: 44-161-446-3036; Fax: 44-161-446-3109; E-mail: ksimpson@picr.man.ac.uk or cdive@picr.man.ac.uk.

doi:10.1158/1078-0432.CCR-08-0111

exhibited single-agent activity *in vivo* in human tumor xenograft models of B-cell lymphoma and small-cell lung carcinoma (6). The impressive antitumor activity *in vivo* was shown in mice bearing xenografts of a range of small-cell lung cancer cell lines, including H146, wherein ABT-737 induced complete regression of 77% H146 tumors when dosed daily at 100 mg/kg/d for 21 days (6). Here, we examine the utility of circulating forms of cytokeratin 18 as blood-borne biomarkers of ABT-737–driven tumor cell death by exploiting the well-established, ABT-737–sensitive H146 small-cell lung cancer tumor model.

The potential of cytokeratins as circulating biomarkers of epithelial cell death resides in the knowledge that they are not expressed in hematopoietic cells. Cytokeratins are expressed in most epithelial cells and in many carcinomas (20, 21), and fragmented or complexed cytokeratins have been detected in the circulation of patients with epithelial malignancies wherein they have been evaluated as tumor biomarkers (20–23). The M65 and M30 ELISAs detect intact and caspase-cleaved forms of cytokeratin 18 (Fig. 1). The M65 assay detects full-length and caspase-cleaved cytokeratin 18 (24) and, as such, is proposed as a biomarker of caspase-dependent and caspase-independent cell death. The M30 assay detects only a cytokeratin 18 neoepitope generated following caspase cleavage at position 387 to 396 and is considered to be a specific assay for epithelial apoptosis (25–27). Several reports propose that the levels of caspase-cleaved cytokeratin 18 are predictive of tumor response to drug treatment (28) and may have prognostic significance (29).

M30 and M65 data presented here show that cleaved and intact cytokeratin 18 are indeed useful blood-borne biomarkers of ABT-737–induced tumor cell death and of tumor burden *per se* because significant correlations between the levels of these circulating biomarkers, tumor apoptosis, and tumor regression were established. This study also showed that these circulating biomarkers confirmed the absence of ABT-737–induced epithelial toxicity following analysis in non–tumor bearing animals treated with ABT-737. These promising preclinical data can now be translated directly to upcoming clinical trials of Bcl-2 family–targeted drugs in epithelial tumors.

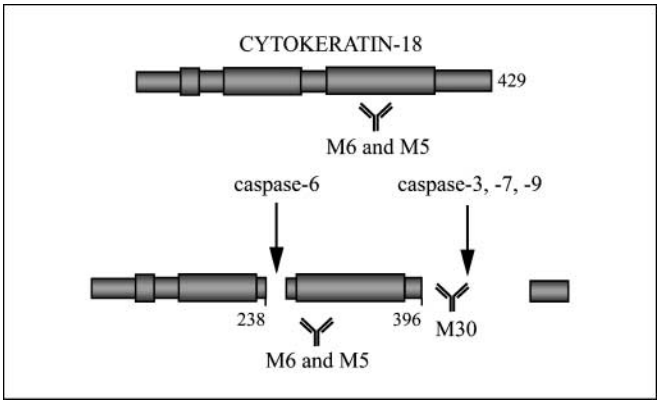

Fig. 1. Schematic representation of cytokeratin 18 caspase cleavage and the sites for M30 and M65 antibody recognition. During apoptosis, activated caspase-3, caspase-6, caspase-7, and caspase-9 are able to cleave cytokeratin 18 at specific peptide recognition sites. Caspase cleavage generates a neoepitope that can be detected using the M30 and M65 assays, thus informing on the levels of apoptosis. In addition, the M65 antibody is also able to detect full-length (intact) cytokeratin 18 and thus provide information on the levels of necrotic cell death. The M65 ELISA uses the M6 antibody as the catcher antibody and M5 as the detection antibody. The M30 ELISA uses M5 as the capture antibody and M30 as the detection antibody.

Materials and Methods

Cell culture. H146 cells were purchased from American Tissue Type Collection and were cultured in RPMI supplemented with 10% FCS, 1% sodium pyruvate, and 4.5 g/L glucose in a 37°C humidified 5% CO_2 incubator and routinely checked for *Mycoplasma* infection.

H146 xenograft studies. All *in vivo* studies were conducted as described previously (6) in accordance with guidelines established by the internal Institutional Animal Care and Use Committee. Female C.B.-17 severe combined immunodeficient (SCID)/*bg* mice were inoculated s.c. with 5×10^6 NCI-H146 small-cell lung cancer cells and tumor size was monitored by caliper measurements ($V = L \times W^2 / 2$). All mice were survival bled via a retro-orbital method 24 h before the first drug dose, and blood was collected in heparin-coated tubes for determination of baseline antigen level for M65 and M30. Blood was processed to isolate plasma that was stored immediately at -70°C. When average tumor volumes reached ~400 mm^3, mice were treated daily with i.p. administration of ABT-737 at 100 mg/kg or vehicle control. ABT-737 was formulated in 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water; pH 4-5). A final terminal bleed was conducted at the indicated time points with concomitant harvest of tumor tissue in 10% neutral buffered formalin. Plasma samples were placed in dry ice while in transit from Abbott Laboratories to the Paterson Institute for Cancer Research, and upon receipt, they were stored at -80°C before analysis within 1 wk.

Detection of M65 and M30 antigens. The M65 and M30 (Apoptosense) ELISA kits (Peviva) were used for the plasma biomarker analysis (3, 4, 30), incorporating a blocking agent (HBR) modification to permit the maintenance of assay dynamic range in a mouse background. HBR functions by binding and removing the cross-reacting heterophilic antibodies, which are thought to interfere with the sandwich ELISA by interacting with the capture and the detection antibodies, thus leading to false-positive amplification of signal. In brief, 25 μL of each sample (standard, quality control, or plasma sample) were added to each well of a 96-well plate coated with the mouse monoclonal "catcher antibody." Following this, 75 μL of horseradish peroxidase–conjugated "detection monoclonal antibody" and 4 μL of the protein blocking agent HBR plus (Scantibodies Laboratory, Inc.) were added per well, and samples were incubated at room temperature for 2 h (M65) or 4 h (M30), followed by removal of excess conjugate. After adding 200 μL of 5.5′-tetramethylbenzidine substrate and incubating 20 min in the dark, 50 μL of 1.0 mol/L sulfuric acid were added and the absorbance was read at 540 nm. The concentration (units/L) of antigen was calculated based on a standard curve from known antigen concentrations. The dynamic ranges of the two ELISAs are 0 to 1,000 units/L and 0 to 2,000 units/L for the M30 and M65 assays, respectively. However, values <20 units/L are considered at the limit of detection for both assays, and data lower than this limit are excluded.

To account for natural biological variations, a baseline measurement of M30 and M65 antigen was recorded for each individual mouse 24 hours before receiving either drug or vehicle. These values were then subtracted from subsequent readings for each individual mouse before grouping animals for statistical analysis. Mann-Whitney *U* test, in addition to one-way ANOVA, followed by a *post hoc* Bonferroni multiple range test (to determine where the differences were among multiple groups), was conducted on all predose samples to test for significant differences in predose data within and between time points for each treatment group.

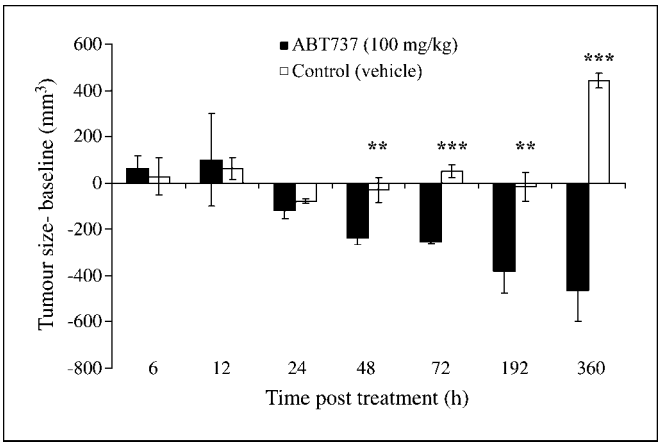

Fig. 2. Effects of ABT-737 and vehicle on H146 human small-cell lung cancer xenograft size. H146 small-cell lung cancer cells were implanted into SCID/*bg* mice and, when tumors reached ~400 mm^3, were treated with either ABT-737 at 100 mg/kg/d for 7 d (*black columns*) or vehicle (*white columns*). Columns, are mean (3 mice); bars, SD. **, $P < 0.01$; ***, $P < 0.001$.

Detection of Cleaved Caspase-3 and Caspase-Cleaved Cytokeratin 18 in Tumor

Cleaved caspase-3. Paraffin-embedded tumor tissue was cut in 3-μm sections, which were deparaffinized and dehydrated. Sections were then microwaved for 25 min in citric acid buffer (10 mmol/L; pH 6.0), followed by the blocking of endogenous peroxidase by immersing in a 0.3% hydrogen peroxide solution for 30 min. The slides were incubated with 10% casein solution for 1 h to remove background staining. Slides were then incubated overnight with the primary antibody [anti–cleaved caspase-3 (Asp^{175}) antibody (9661S, Cell Signalling)] at 4°C in a humidified tray. After incubation, slides were washed in PBS and goat anti–rabbit (Vectastain ABC kit from Vecta Laboratories, Inc.; reference, PK-4001) secondary antibody was added, respectively, for 30 min, followed by PBS wash. ABC kit (Envision Kit, Vector Laboratories) to amplify signal was applied (according to the manufacturer's instructions), and sections were washed in PBS before the visualization of signal using 3,3′-diaminobenzidine reagent (Dako K4011).

Caspase-cleaved cytokeratin 18. The sections were cut and collected on Surgipath Xtra adhesive slides and were then dried overnight at 37°C, followed by 10 min at 60°C. After dewaxing, antigen retrieval was achieved by heating at 95°C for 12 min, followed by cooling in Dako (S3307) high-pH retrieval solution. The following steps were done using the i6000 automated immunohistochemistry platform. Hydrogen peroxide solution (3%) was applied for 10 min. Blocking was carried out by using the affinity purified goat anti-mouse Fab fragment (Jackson 115-007-003) for 15 min at room temperature, washing, and then incubating with 5% casein solution for 20 min. The primary M30 Cytodeath antibody (Peviva, 10700) was applied for 120 min at room temperature, followed by a 30-min incubation with the α mouse envision-labeled polymer (Dako K4006), and eventually, the signal was visualized using the 3,3′-diaminobenzidine reagent. All sections were counterstained with hematoxylin so that negative cells can be identified, followed by dehydration in increasing concentrations of ethanol solution (70%, 90%, 100%) for 1 min and xylene for 5 min before mounting.

Slides were analyzed blindly by two independent scorers. Sections were chosen randomly for counting, but areas containing overt necrosis that gave nonspecific staining (universally brown-stained cells when using immunoglobulin isotype control) were unquantifiable and avoided. The number of positive cells was determined as the mean from five independent fields on each section, and statistical significance was determined using a two-tailed Student's t test. Results were expressed as mean ± SE.

Results

Preclinical studies were carried out using SCID/*bg* mice that were either non–tumor bearing or carried an H146 human small-cell lung cancer tumor xenograft. Tumor and non–tumor-bearing mice were either treated with ABT-737 (100 mg/kg/d) or vehicle control. Blood was taken at various time points during the study and processed to generate plasma samples. Samples were assayed for total cytokeratin 18 (intact and caspase cleaved) using the M65 ELISA and the levels of caspase-cleaved cytokeratin 18 were calculated using the M30 ELISA, both validated assays. Tumors were harvested and stained for biomarkers of apoptosis, cleaved caspase-3, and caspase-cleaved cytokeratin 18 using validated immunohistochemistry protocols.

Regression of H146 small-cell lung cancer tumors after treatment with ABT-737 and growth of control tumors. Figure 2 confirmed that, during this biomarker study and upon commencement of dosing with either ABT-737 or vehicle control, xenografts from mice receiving ABT-737 showed almost complete regression after 192 hours (8 days), an effect that was maintained for the duration of the study (15 days). In contrast, mice receiving vehicle control maintained relatively stable tumor sizes up to 8 days, but by the end of the study, these tumors were significantly larger than ABT-737–treated tumors and had reached >800 mm^3 ($P < 0.01$ 48 hours, $P < 0.001$ 72 hours, $P < 0.01$ 8 days, and $P < 0.001$ 15 days; tumor volume ABT-737–treated versus vehicle-treated animals).

Levels of biomarkers of epithelial cell death in tumor-bearing mice treated with ABT-737. To conclude that significant changes in M30 and M65 antigen levels in tumor-bearing mice treated with ABT-737 compared with those receiving vehicle were a result of treatment, comprehensive statistical evaluation was carried out. Predose data were compared across all time points using one-way ANOVA, followed by a *post hoc* Bonferroni analysis to adjust for multiple sampling within groups, and Mann-Whitney *U* testing was used to identify

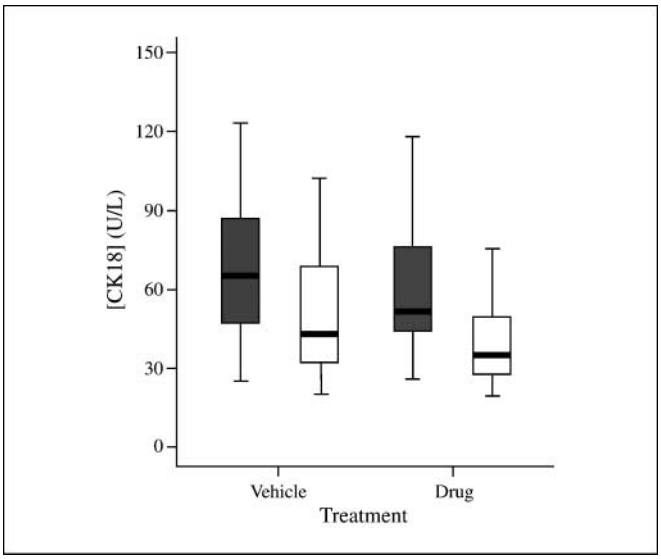

Fig. 3. Variation in predose (baseline) levels of M30 and M65. One-way ANOVA, followed by a *post hoc* Bonferroni analysis was carried out to test for significant variation in 24 h predose (baseline) levels of M30 (*gray columns*) and M65 (*white columns*) antigens in vehicle- and ABT-737–treated animals.

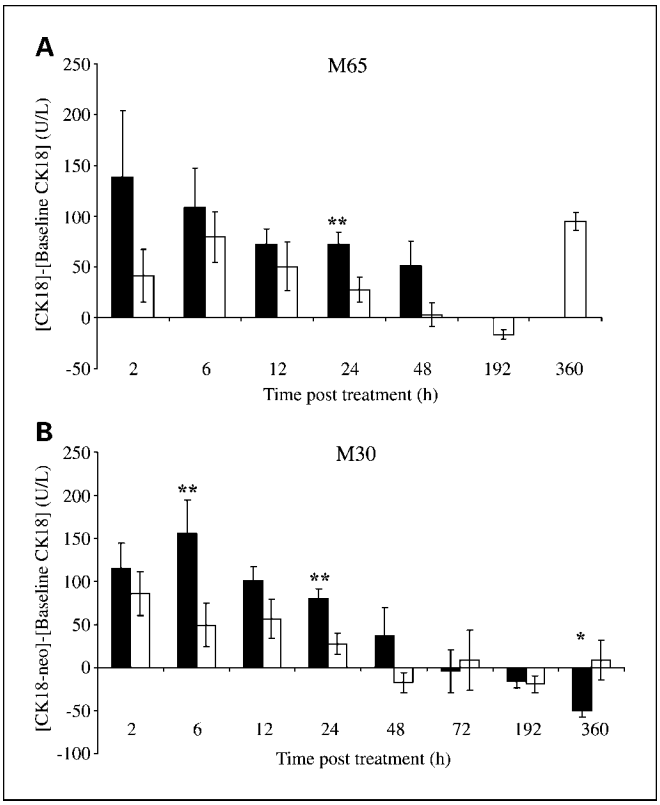

Fig. 4. Levels of circulating cytokeratin 18 (*M65*; *A*) and cytokeratin 18-neo (*M30*; *B*) in H146 small-cell lung cancer human xenograft–bearing mice treated with either ABT-737 or vehicle. Female age- and sex-matched SCID/*bg* mice were implanted with H146 small-cell lung cancer cells until the tumors reached ~400 mm^3, at which point animals received either daily ABT-737 at 100 mg/kg/d via i.p. administration (*black columns*) or vehicle (*white columns*) and terminal blood plasma samples were taken and assayed for intact cytokeratin 18 (*A*) and cleaved cytokeratin 18 (*B*) using M65 and M30 assays, respectively. Levels of cytokeratin 18 or cleaved cytokeratin 18 were calculated by subtraction of baseline levels taken 24 h before dosing via retro-orbital survival bleed. Data are from 10 (6, 12, 24, and 192 h), 7 (2 h), or 3 (48, 72, and 360 h) ABT-737–treated mice per group and 10 (6, 12, 24, and 192 h), 7 (2 h), or 3 (48, 72, and 360 h) vehicle control–treated mice. Columns, mean; bars, SE. *, $P < 0.05$.

differences between groups. Figure 3 shows that there were no statistically significant differences in the predose M30 and M65 levels across both treatment groups, and thus any changes seen post dosing were indeed a result of drug treatment and not a consequence of biological variation.

In agreement with our collaborators at the Karolinska Institute, we have found that the M30 and M65 assays were much less sensitive at detecting endogenous mouse cytokeratin 18 when compared with human cytokeratin 18.[3] As such, baseline levels of circulating cytokeratin 18 were up to 4-fold higher in tumor-bearing compared with non–tumor-bearing mice, indicative of a tumor-derived biomarker signal. In the case of non–tumor-bearing animals treated with ABT-737 or vehicle, M30 and M65 signals were close to or at the lower limit of detection for the assay (20 units/L, data not shown). Changes in M65 (Fig. 4A) and M30 readings (Fig. 4B) were calculated for each individual mouse by subtracting the reading at baseline in that mouse of the circulating antigen measured 24 hours before the first dose of ABT-737. M30 and M65 levels were recorded for individual mice before the receipt of their first dose such that baseline or background levels for these markers could be obtained. Baseline subtraction was carried out to account for effects on M30 and M65 levels due to natural biological variation, making it then possible to more closely monitor drug or tumor-specific changes in cytokeratin 18 (Fig. 4).

Figure 4A shows that, in ABT-737–treated tumor-bearing mice, M65 levels were increased 2 to 48 hours after drug treatment compared with baseline levels and compared with mice receiving vehicle. The M65 signal for ABT-737 treatment groups at 72, 192, and 360 hours was below the limit of detection for the assay (20 units/L), and, thus, no comparison could be made between ABT-737 and vehicle treatment groups at these time points. The lack of M65 signal for these animals is perhaps reflective of the low tumor volumes observed at these time points and that tumors had completely regressed by day 15 (Fig. 2).

Vehicle control–treated tumor-bearing animals also exhibited increased levels of M65 antigen at 2 to 24 hours, although the readings at 24 hours were significantly lower than for ABT-737–treated animals at this time point ($P < 0.01$). Notably, there was no difference in tumor volume for ABT-737– and vehicle control–treated mice up to 24 hours (Fig. 2). The M30 results showed a similar biomarker profile to those for M65 with two notable exceptions (Fig. 4B). The overall difference between the increased levels of M30 antigen 2 to 48 hours after treatment with ABT-737 compared with vehicle control was greater, reaching significance at 6 hours ($P < 0.01$; >3-fold increase) and at 24 hours ($P < 0.01$; >3-fold). In contrast to the M65 data, the M30 antigen levels did not increase for vehicle control at 15 days.

Overall, the M30 data more sensitively reported a drug-induced effect at early time points before clear changes in tumor volume. In particular, Fig. 4A shows that, in tumor-bearing mice, those that have been treated with ABT-737 (*black columns*), the levels of cytokeratin 18 measured by M65 2 hours after dosing were 2- to 3-fold higher than in those mice that received vehicle control (*white columns*), and these levels decreased throughout the course of the experiment as the tumors regressed. The data presented confirm that caspase-cleaved cytokeratin 18 is a major contributor to the M65 signal in this study as anticipated for a "pure" apoptosis inducer. This comparison of M65 and M30 will be of relevance in clinical trials of this type of agent.

Biomarkers of apoptosis in tumor. To relate the circulating biomarkers of cell death (M65 or M30) described above to cell fate within ABT-737–treated tumors, immunohistochemical evaluation of cleaved caspase-3, a classic biochemical measurement of apoptosis, was undertaken throughout the time course of the study (Fig. 5). Figure 5A shows that, at 6 to 24 hours posttreatment, the levels of cleaved caspase-3 increased 3- to 4-fold in sections of ABT-737–treated versus vehicle control–treated tumors ($P < 0.05$, 2 hours; $P < 0.001$, 6, 12, and 24 hours). After 8 days, cleaved caspase-3 staining returned to a similar level to that which was seen in the vehicle control– and ABT-737–treated tumors at 2 hours. In addition to this, immunohistochemical analysis of caspase-cleaved cytokeratin 18 could be directly assessed in the tumor using the M30 antibody (Fig. 5B). Although the absolute

[3] M. Hägg-Olofsson, J. Cummings, W. Fayad, S. Brnjic, R. Herrmann, M. Berndstsson, C. Hodgkinson, E. Busk, C. Dive and S. Linder. Determining tumour cell death in the plasma of xenograft-bearing rodents. Cancer Biomarkers, 2008, in press.

numbers of M30-positive tumor cells were low (perhaps reflecting the later stage in apoptosis before phagocytic removal of apoptotic cells), this is not an uncommon feature of M30 immunohistochemistry (32) and showed that levels of caspase-cleaved cytokeratin 18 were higher in xenografts from animals treated with ABT-737 compared with animals that had received vehicle and were significantly higher after 15 days ($P < 0.05$). The kinetics corroborate the theory that caspase-3 activation occurs at an earlier time point during the onset of apoptosis, after which, it is possible to monitor the cleavage of downstream targets (in this case, cytokeratin 18) at later time points. Figure 5 therefore corroborates results shown for circulating biomarkers of cell death in tumor-bearing mice that had received ABT-737 (Fig. 4) and shows an ABT-737 tumor-specific apoptosis.

Levels of circulating biomarkers of epithelial cell death in non–tumor-bearing mice treated with ABT-737. The contribution of host cell death to the biomarker signatures obtained above was explored in a study on ABT-737 non–tumor-bearing mice using a protocol that included positive quality assurance controls. ABT-737 treatment resulted in no significant increase in the circulating levels of intact cytokeratin 18 or cleaved cytokeratin 18 in the plasma of non–tumor-bearing mice when compared with baseline levels taken 24 hours before treatment (data not shown). These data suggest that either ABT-737 exhibits no epithelial host toxicity measurable with these assays or that these assays do not detect mouse cytokeratin 18. To examine this further, we treated mice with a dose of cisplatin (10 mg/kg i.p.) known to induce epithelial toxicity associated with observed animal weight loss and again saw no significant change in M30 or M65 in plasma (data not shown). Overall, these data suggest that the M30 and M65 assays do not detect mouse cytokeratin 18, and, thus, the biomarker data obtained for tumor-bearing mice is derived from the human tumor xenograft.

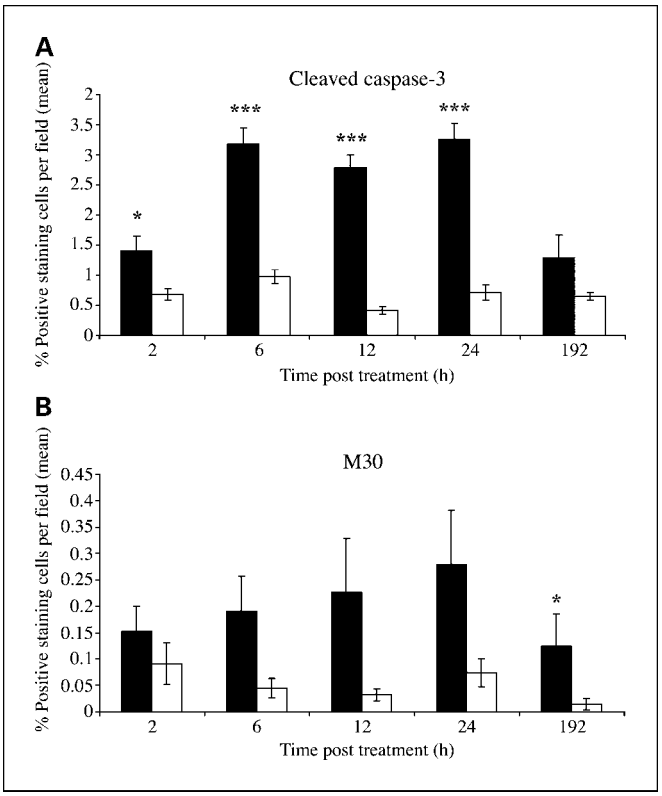

Fig. 5. Analysis of biomarkers of apoptosis in H146 small-cell lung cancer xenograft sections from mice treated with either ABT-737 or vehicle. SCID/*bg* mice were implanted with H146 small-cell lung cancer cells and when tumors reached ~400 mm^3 were treated with either ABT-737 at 100 mg/kg/d for 7 d (*black columns*) or vehicle control (*white columns*) and immunohistochemical analysis of the number of cells that scored positive for cleaved caspase-3 (*A*) or caspase-cleaved cytokeratin 18 (M30; *B*) was determined between 2 and 192 h after dosing commenced. Data are from seven ABT-737– and seven vehicle-treated mice per time point and are shown as the average from five fields of view from each tumor section as determined by two independent analysts who were blinded to tumor group identity. Columns, mean; bars, SE.

Discussion

Avoidance of apoptosis is a hallmark of cancer (32) and novel agents that target components of the apoptotic pathway are currently in preclinical and early clinical development. Selective tumor cell kill by these proapoptotic drugs is anticipated because, unlike normal cells, cancer cells exist in hostile microenvironments that prime them for apoptosis. The premise is that such cancer cells survive because their adaptive up-regulation of antiapoptotic proteins, such as members of the Bcl-2 and IAP families, maintain inability to couple stress-inducing stimuli to the activation of apoptosis. Bcl-2 family–targeted agents such as ABT-737, a highly potent and specific inducer of apoptosis *in vitro* and in preclinical models *in vivo* (6), are entering phase I or II trials; therefore, qualified pharmacodynamic biomarkers of drug-induced apoptosis are required as the hypothesis underlying selective tumor cell killing begins to be tested in patients with cancer.

The cytokeratin 18–based M30 and M65 assays have been used in the clinic to monitor cell death induced by a variety of different cancer chemotherapeutic agents in a range of malignancies (3). Although the M30 and M65 ELISAs have been validated *in vitro* (3, 4, 30), the levels of circulating forms of cytokeratin 18 as biomarkers of prognosis and treatment response in patients have yet to be qualified. The studies have thus far largely supported the use of circulating levels of cleaved cytokeratin 18 to inform on tumor cell apoptosis and thus determine treatment response (29, 33, 34), and several reports recently suggested that levels of circulating caspase-cleaved cytokeratin 18 correlate with poor survival rates in some cancers (6, 28, 29). Apoptotic tumor cells have been shown in the blood using the M30 antibody (35); yet, it remains unclear about whether most caspase-cleaved cytokeratin 18 present in the blood of drug-treated cancer patients derives from tumor or from other epithelial sites as a result of drug toxicity or secondary effects of the malignancy (5).

This study represents the first assessment of the M30 and M65 assays in response to an apoptosis-targeted drug in a preclinical *in vivo* setting. Because only animals whose tumors were responding to ABT-737 treatment were included in the study, biomarker data were not affected by nonresponders. The rationale behind this preclinical study was therefore to inform on the validity of M30 and M65 levels as circulating biomarkers of tumor cell death in a tumor model with known sensitivity to the Bcl-2 antagonist ABT-737, where a parallel assessment of tumor cell death with the circulating biomarkers could be done. In addition, it was envisaged that this study could provide

information on optimum time points for blood sampling from clinical trial patients receiving BH-3 mimetics such as ABT-737 to allow kinetics of tumor cell death to be inferred from circulating biomarker profiles with some confidence when serial tumor biopsies were unavailable for analysis.

This type of study was previously hampered by technical issues presented by the high background signal in mouse blood due to cross-reactivity between mouse immunoglobulins and the mouse antibodies used in these assays. Here, this is overcome through use of a heterophilic blocking reagent that minimizes the high background signal often seen in mouse plasma samples.

In this study, cleaved cytokeratin 18 levels in plasma were higher in animals receiving drug, compared with those receiving vehicle control, with a drug-specific effect apparent as early as 6 hours after receiving ABT-737 (Fig. 4B). Figure 4B shows a statistically significant difference between M30 levels in ABT-737–treated and control mice at 6 and 24 hours ($P < 0.01$) after dosing. That these levels decline over the course of the experiment and that this correlates with tumor regression (Fig. 2) strongly suggest that ABT-737 is indeed causing cell death by apoptosis, and the levels of cleaved cytokeratin 18 in the blood is indicative of a drug response.

In contrast, M65 levels in animals with small-cell lung cancer xenografts receiving ABT-737 were not significantly different compared with those that received vehicle at very early time points (6 hours). However, after 24 hours, the levels of M30 and M65 were significantly higher in animals receiving ABT-737 compared with vehicle control–treated mice ($P < 0.01$). This suggests that the rapid kinetics of ABT-737–induced tumor regression by apoptosis may progress to secondary necrosis at later times (24 hours). This observation is consistent with the proposed mechanism of action of the drug and is corroborated by an agreement of drug-induced changes in M65 and M30, which suggests that cell death occurs predominantly via an apoptotic mechanism.

Of particular note, the M30 and M65 assays were able to detect changes in circulating biomarkers in mice with small-cell lung cancer xenografts up to 24 hours after drug treatment, whereas changes in tumor volume were not significantly different until 48 hours after receiving ABT-737 ($P < 0.01$). This is an important consideration when assessing the utility of a biomarker in the clinic. Indeed, these preclinical data suggest that the M30 and M65 blood-borne assays may provide a valuable tool for detecting early tumor response to apoptosis-inducing therapy that we speculate could occur before discernable changes in tumor volume measured by imaging and thus may ultimately have use in predicting an appropriate time point for imaging patients.

Although it is known that ABT-737 treatment induces an apoptosis-like response in platelets, resulting in enhanced platelet clearance by the reticuloendothelial system (36), the M30 and M65 assays would not detect this hematopoietic cytotoxicity. The finding that the levels of M30 and M65 antigen are increased in tumor-bearing mice compared with control and non–tumor-bearing mice and that significant changes in the levels of circulating cytokeratin 18 occurs only in ABT-737–treated animals bearing xenografts provide further compelling evidence that cytokeratin 18 measured via this method is a reliable measure of events occurring within the tumor. This is consistent with data in Fig. 5 that showed both caspase-3 and cytokeratin 18 cleavage to be elevated in tumors receiving ABT-737 treatment. The lack of biomarker signals in non–tumor-bearing mice treated with a dose of cisplatin known to provoke epithelial toxicity and weight loss further supports that the circulating cytokeratin 18 biomarker signatures seen in ABT-737–treated mice are derived from the human tumor xenograft. Complementing this study, we have recently published a corresponding induction of drug-induced apoptosis in human xenograft tumor measured by M30 and reported in plasma with M30 in a nude rat model (32).

The kinetics of apoptosis caused by ABT-737 as detected by the M30 and M65 assays in this study is consistent with other studies of ABT-737 responses *in vitro* whereby maximal caspase-3 activity was seen 6 to 24 hours after treatment (15, 18, 19), followed by the degradation of cytokeratin 18 to generate the caspase-cleaved neoepitope recognized by M30 after 192 hours. Similarly, the kinetics of ABT-737–induced release of cytokeratin 18 from dying cells is in agreement with other clinical studies wherein elevated levels of circulating intact and cleaved cytokeratin 18 were seen in plasma following conventional chemotherapeutic treatment of lung (29), hormone refractory prostate (24), and breast cancers (28, 33) between 24 to 48 hours posttreatment. These observations are all consistent with the primary mechanism of action of the drug being induction of apoptosis.

A recent 2007 review by Linder (5) highlighted the issue that the measurement of circulating caspase-cleaved cytokeratin 18 (M30) in patient samples is a promising method to determine the efficiency of cytotoxic drug treatment and could also be used to compare these with novel therapies. However, the author also stated that the use of M30 for monitoring treatment response in individual patients is yet to be unequivocally shown. Further clinical studies are required to assign the use of M30 as a biomarker in the clinic. However, this is the first example of a preclinical study that should provide strength to the validity of the clinical use of the M30 and M65 assays because changes in tumor volume directly correlate with changes in circulating biomarkers of cell death.

In light of this, the data shown here for a pure apoptosis inducer, ABT-737, a drug targeted directly at apoptosis regulatory machinery, provide further evidence for the use of M30 as an indicator of drug-induced apoptosis that, in this model, occurred predominantly in tumor cells and correlated with tumor response. This study has proved highly informative both in terms of the validation of the M30 and M65 ELISAs and as a reliable means to provide pharmacodynamic information about tumor cell death following treatment *in vivo* and to provide substantiating evidence for the mode of action *in vivo* for the novel antiapoptotic agent, ABT-737. Furthermore, we have obtained valuable information about the kinetics for measurement of cell death products in the blood, which should prove valuable for the detailed and optimal assessment of this promising novel anticancer agent in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank C. Hodgkinson and A. Hogg for technical support.

References

1. Workman P. Cancer genome targets: RAF-ing up tumor cells to overcome oncogene addiction. Expert Rev Anticancer Ther 2002;2:611–4.
2. Taylor K, Micha D, Ranson M, Dive C. Recent advances in targeting regulators of apoptosis in cancer cells for therapeutic gain. Expert Opin Investig Drugs 2006;15:669–90.
3. Cummings J, Ranson M, Butt F, Moore D, Dive C. Qualification of M30 and M65 ELISAs as surrogate biomarkers of cell death: long term antigen stability in cancer patient plasma. Cancer Chemother Pharmacol 2007;60:921–4.
4. Cummings J, Ward TH, Greystoke A, Ranson M, Dive C. Biomarker method validation in anticancer drug development. Br J Pharmacol 2007;153:646–56.
5. Linder S. Cytokeratin markers come of age. Tumour Biol 2007;28:189–95.
6. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677–81.
7. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 2003;22:8590–607.
8. Petros AM, Olejniczak ET, Fesik SW. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta 2004;1644:83–94.
9. Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. Cancer Res 2007;67:782–91.
10. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell 2006;10:375–88.
11. Tahir SK, Yang X, Anderson MG, et al. Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. Cancer Res 2007;67:1176–83.
12. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006;10:389–99.
13. Cragg MS, Kuroda J, Puthalakath H, Huang DC, Strasser A. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. PLoS Med 2007;4:1681–89; discussion 90.
14. Gong Y, Somwar R, Politi K, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. PLoS Med 2007;4:e294.
15. Kuroda J, Kimura S, Strasser A, et al. Apoptosis-based dual molecular targeting by INNO-406, a second-generation Bcr-Abl inhibitor, and ABT-737, an inhibitor of antiapoptotic Bcl-2 proteins, against Bcr-Abl-positive leukemia. Cell Death Differ 2007;14:1667–77.
16. Shoemaker AR, Oleksijew A, Bauch J, et al. A small-molecule inhibitor of Bcl-XL potentiates the activity of cytotoxic drugs *in vitro* and *in vivo*. Cancer Res 2006;66:8731–9.
17. Trudel S, Stewart AK, Li Z, et al. The Bcl-2 family protein inhibitor, ABT-737, has substantial antimyeloma activity and shows synergistic effect with dexamethasone and melphalan. Clin Cancer Res 2007;13:621–9.
18. Kline MP, Rajkumar SV, Timm MM, et al. ABT-737, an inhibitor of Bcl-2 family proteins, is a potent inducer of apoptosis in multiple myeloma cells. Leukemia 2007;21:1549–60.
19. Kohl TM, Hellinger C, Ahmed F, et al. BH3 mimetic ABT-737 neutralizes resistance to FLT3 inhibitor treatment mediated by FLT3-independent expression of BCL2 in primary AML blasts. Leukemia 2007;21:1763–72.
20. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. Histopathology 2002;40:403–39.
21. Lane EB, Alexander CM. Use of keratin antibodies in tumor diagnosis. Semin Cancer Biol 1990;1:165–79.
22. Hatzfeld M, Franke WW. Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombinations of purified polypeptides. J Cell Biol 1985;101:1826–41.
23. Steinert PM, Roop DR. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 1988;57:593–625.
24. Kramer G, Erdal H, Mertens HJ, et al. Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. Cancer Res 2004;64:1751–6.
25. Biven K, Erdal H, Hagg M, et al. A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera. Apoptosis 2003;8:263–8.
26. Leers MP, Kolgen W, Bjorklund V, et al. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 1999;187:567–72.
27. Schutte B, Henfling M, Kolgen W, et al. Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res 2004;297:11–26.
28. Demiray M, Ulukaya EE, Arslan M, et al. Response to neoadjuvant chemotherapy in breast cancer could be predictable by measuring a novel serum apoptosis product, caspase-cleaved cytokeratin 18: a prospective pilot study. Cancer Invest 2006;24:669–76.
29. Ulukaya E, Yilmaztepe A, Akgoz S, Linder S, Karadag M. The levels of caspase-cleaved cytokeratin 18 are elevated in serum from patients with lung cancer and helpful to predict the survival. Lung Cancer 2007;56:399–404.
30. Cummings J, Ward TH, LaCasse E, et al. Validation of pharmacodynamic assays to evaluate the clinical efficacy of an antisense compound (AEG 35156) targeted to the X-linked inhibitor of apoptosis protein XIAP. Br J Cancer 2005;92:532–8.
31. Hagg-Olofsson M, Cummings J, Fayad W, et al. Determining tumour cell death in the plasma of xenograft-bearing rodents, Cancer Biomarkers. In press 2008.
32. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
33. Olofsson MH, Ueno T, Pan Y, et al. Cytokeratin-18 is a useful serum biomarker for early determination of response of breast carcinomas to chemotherapy. Clin Cancer Res 2007;13:3198–206.
34. Ueno T, Toi M, Biven K, Bando H, Ogawa T, Linder S. Measurement of an apoptotic product in the sera of breast cancer patients. Eur J Cancer 2003;39:769–74.
35. Larson CJ, Moreno JG, Pienta KJ, et al. Apoptosis of circulating tumor cells in prostate cancer patients. Cytometry A 2004;62:46–53.
36. Zhang H, Nimmer PM, Tahir SK, et al. Bcl-2 family proteins are essential for platelet survival. Cell Death Differ 2007;14:943–51.

Clinical Cancer Research

Circulating Biomarkers of Cell Death After Treatment with the BH-3 Mimetic ABT-737 in a Preclinical Model of Small-Cell Lung Cancer

Dimitra Micha, Jeff Cummings, Alex Shoemaker, et al.

Clin Cancer Res 2008;14:7304-7310.