ABT-737 Overcomes Resistance to Immunotoxin-Mediated Apoptosis and Enhances the Delivery of Pseudomonas Exotoxin–Based Proteins to the Cell Cytosol

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Abstract

Pseudomonas exotoxin (PE)–based immunotoxins (antibody-toxin fusion proteins) have achieved frequent complete remissions in patients with hairy cell leukemia but far fewer objective responses in other cancers. To address possible mechanisms of resistance, we investigated immunotoxin activity in a model system using the colon cancer cell line, DLD1. Despite causing complete inhibition of protein synthesis, there was no evidence that an immunotoxin targeted to the transferrin receptor caused apoptosis in these cells. To address a possible protective role of prosurvival Bcl-2 proteins, the BH3-only mimetic, ABT-737, was tested alone or in combination with immunotoxins. Neither the immunotoxin nor ABT-737 alone activated caspase 3, whereas the combination exhibited substantial activation. In other epithelial cell lines, ABT-737 enhanced the cytotoxicity of PE-related immunotoxins by as much as 20-fold, but did not enhance diphtheria toxin or cycloheximide. Because PE translocates to the cytosol via the endoplasmic reticulum (ER) and the other toxins do not, ABT-737–mediated effects on the ER were investigated. ABT-737 treatment stimulated increased levels of ER stress response factor, ATF4. Because of its activity in the ER, ABT-737 might be particularly well suited for enhancing the activity of immunotoxins that translocate from the ER to the cell cytosol.

Introduction

Immunotoxins are antibody-toxin chimeric proteins targeted to kill cancer cells (1–3). Design features include an antibody or antibody fragment and a toxin moiety that is directed to kill specific cells displaying a surface receptor or antigen. Recombinant immunotoxins composed of antibody Fvs linked to domains II and III of Pseudomonas exotoxin (PE) have shown great promise for the treatment of hematologic malignancies but have done less well against cancers derived from epithelial cells (4–9). A feature of protein immunotoxins is the potency associated with the enzymatic component of the toxin. PE and diphtheria toxin (DT) are both cytotoxic because they gain access to the cytosol and inhibit protein synthesis via the ADP-ribosylation of EF2. However, there is a significant difference in the intracellular route taken by each toxin. DT uses the low pH of endosomes to escape from this organelle to the cytosol (10, 11). PE does not translocate from endosomes but rather uses a KDEL (Lys-Arg-Glu-Leu) retrieval sequence at its COOH terminus to traffic to the endoplasmic reticulum (ER). Once there, an active fragment translocates to the cytosol, possibly using the endoplasmic reticulum-associated degradation system (12–15). In the cytosol, both toxins act catalytically to ADP-ribosylate EF2 and shut down protein synthesis. It has been shown that one or only a few of these toxin molecules delivered to the cytosol are sufficient to kill a target cell (16). Much of that argument is based on early experiments in which killing was equated solely with the inhibition of protein synthesis. Recently, assays that focus more precisely on the mechanisms of cell death have been developed. These have led to the appreciation that some growth-inhibitory treatments, whereas apparently causing biochemical damage, do not result in efficient death (17). Resistance to apoptosis is an important reason why cells fail to die. In part, this is because prosurvival proteins, such as those within the BCL-2 family of proteins, could keep cells alive even in the face of harsh treatments. In fact, the basal level expression of Bcl-xl was the only 1 of 10 candidate genes matched with 122 standard...
chemotherapy agents that negatively correlated with sensitivity in the NCI 60 cell line screen (18).

ABT-737 and ABT-263 were developed as agents to neutralize the prosurvival effects of Bcl-2 proteins (19, 20). Because ABT-737 is a BH3-only mimic, it can bind to the hydrophobic groove in Bcl-2 proteins and liberate Bax or Bak allowing the process of apoptosis to proceed efficiently. Binding experiments have revealed that ABT-737 has high affinity for Bcl-2, Bcl-xl, and Bcl-w, but little or no affinity for Mcl-1 (21). Thus, in cancers in which Mcl-1 is a pivotal prosurvival protein, ABT-737 is less effective (21, 22). Several reports therefore suggest that combination treatments of ABT-737 with agents that degrade or neutralize Mcl-1 have a better chance of being effective compared with ABT-737 alone (23–25). Mcl-1 has a short half-life of ~30 minutes. Agents that inhibit protein synthesis will lead to the loss of Mcl-1 over time (26, 27). Thus, by combining ABT-737, an agent that neutralizes three major BCL2 proteins, and an immunotoxin, an agent that causes a loss in Mcl-1, apoptosis might be achieved selectively in cells displaying target antigens.

Toxin and immunotoxin activities have been associated with apoptosis in some cell systems but the mechanisms of cell death have not been extensively studied (26, 28–32). To study immunotoxin-cell outcomes, we have used two PE-based immunotoxins, HB21-PE40 (directed to the human transferrin receptor; ref. 33) and SS1P (directed to density of 5 × 10⁴/mL. The next day, cells were treated follows: KB3-1 cells were seeded in six-well plates at a

**Materials and Methods**

**Compounds**

ABT-737 was purchased from Selleck Chemicals LLC, dissolved in DMSO at 10 mmol/L stock concentration, and stored frozen at −20°C. ABT-263 was obtained from Toronto Research Chemicals, Inc., dissolved in DMSO at 3 mmol/L, and stored frozen at −20°C. Velcade (bortezomib) was obtained from the NIH pharmacy. HB21-PE40 and SS1P were produced recombinantly in *Escherichia coli* as described previously (33, 34). HB21-CET40 was described recently (35). DT was purchased from List Biological Laboratories, PE was from laboratory stocks, and pyruvate. The KB3-1 cells were obtained from Michael Gottesman (National Cancer Institute, Bethesda, MD), and grown in DMEM plus 10% fetal bovine serum.

**Antibodies**

PARP (BD; 556494), caspase 3 (Santa Cruz Biotechnology; 7148), Mcl-1 (Cell Signaling; 4572), tubulin (Sigma; T6074), and ATF4 (Santa Cruz Biotechnology; SC-200).

**Cells**

DLD1 and SKOV3 were obtained from American Type Culture Collection and were each grown in RPMI 1640 plus 10% fetal bovine serum, pen-strep, and pyruvate. The KB3-1 cells were obtained from Michael Gottesman (National Cancer Institute, Bethesda, MD), and grown in DMEM plus 10% fetal bovine serum.

**Assays**

Treated cells were assayed for inhibition of protein synthesis by the addition of ³H-leucine (2 μCi/mL) for 4 hours in 96-well plates. Cells were collected on filter mats and samples counted using a Wallac beta plate reader. To measure cytotoxicity, water-soluble tetrazolium-1 (WST-1; Roche) was added to 96-well plates at a final concentration of 10% and absorbance measured at 450 nm. ATP assays were conducted using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). Caspase 3/7 was detected using the Caspase-Glo kit from Promega. Caspase 3 activity was measured using one of two fluorescent substrates Caspase 3 Fluorometric Assay Kit (R&D Systems) or Caspase 3 Fluorometric-KIT (Invitrogen). Data are reported in fluorescent units per microgram of cell protein. A short-exposure cell-killing assay was carried out as follows: KB3-1 cells were seeded in six-well plates at a density of 5 × 10⁴/mL. The next day, cells were treated for 4 hours with either immunotoxin alone, ABT-737 alone, or combinations of both as indicated. At the end of the treatment, cells were washed with PBS, trypsinized, and replated. Replated cells were incubated for an additional 6 days. Finally, cells were washed with PBS and then stained with methylene blue (0.5%) in methanol/water (50:50 by volume)

**Western blots**

Immunotoxin-treated cells in the presence or absence of ABT-737 were washed with PBS and then solubilized with radiolabeled precipitation assay buffer containing both protease and phosphatase inhibitors. Precast Tris-glycine 8% to 16% gels were used to separate cell lysates. Primary antibodies were routinely detected with donkey anti-mouse horseradish peroxidase or donkey anti-rabbit horseradish peroxidase (Jackson ImmunoResearch).

**Results**

**ABT-737 overcomes resistance to immunotoxin-mediated apoptosis in DLD1 cells**

Resistance to apoptosis is a major obstacle to effective cytotoxic cancer therapy (18). Colorectal cancer cells are among the most difficult to kill (36). DLD1 cells (a colon cancer cell line) are susceptible to growth inhibition (36), which is most likely due to the human transferrin receptor (HB21-PE40). HB21-PE40 inhibited protein synthesis of DLD1 cells in a dose-response manner with complete inhibition being achieved at concentrations of, or exceeding, 1 ng/mL (Fig. 1A). In cytotoxicity...
assays, HB21-PE40 was active with an IC50 of ∼0.1 ng/mL (Fig. 1B and C). However, using either a WST-1 or an ATP depletion assay, there was incomplete killing at higher concentrations (Fig. 1B and C). Furthermore, when cells were treated with HB21-PE40 (10 ng/mL) for 72 hours and then monitored for viability, ∼20% of cells excluded trypan blue, further indicating incomplete killing (data not shown). When caspase 3/7 activity was measured, immunotoxin-treated DLD1 cells showed no evidence of apoptosis (Fig. 1D). However, apoptosis, as evidenced by increased caspase 3/7 activity, was noted when velcade (a proteasome inhibitor) was added as a positive control (Fig. 1D). We conclude that DLD1 cells are resistant to immunotoxin-mediated apoptotic death.

To address the role of prosurvival Bcl-2 proteins in mediating resistance, we investigated the outcome of combining the BH-3-only mimetic, ABT-737, with immunotoxins. ABT-737 neutralizes Bcl-2, Bcl-xl, and Bcl-w but does not bind Mcl-1. However, agents that inhibit protein synthesis, including immunotoxins, result in the loss of Mcl-1 due to its short half-life (27). Thus, combination treatments of an immunotoxin and ABT-737 could neutralize or eliminate all the relevant prosurvival Bcl-2 family proteins. In Fig. 2A, the addition of ABT-737 increased the cytotoxicity of all concentrations of the HB21-PE40 immunotoxin for DLD1 cells. To investigate the basis for this, we probed elements of the apoptosis pathway. Neither immunotoxin alone (at 1 or 10 ng/mL) nor ABT-737 (at 10 μmol/L) caused a large increase in caspase 3 activity. However, the combination resulted in a substantial increase in activity compared with either treatment alone (Fig. 2B). That activation of caspase 3 was primarily due to immunotoxin-mediated inhibition of protein synthesis, in combination with ABT-737, was confirmed by showing that cycloheximide in combination with ABT-737 produced a similar result (Fig. 2C). Further analysis of key members of the intrinsic apoptosis pathway was conducted. Radioimmunoprecipitation assay buffer extracts were probed for Mcl-1 and cleavage of PARP. Samples from immunotoxin-treated cells exhibited a loss of Mcl-1, whereas only those that were also treated with ABT-737 resulted in the cleavage of PARP (Fig. 2D). Of note was the apparent increase in Mcl-1 levels in samples treated with ABT-737 alone (Fig. 2D, lane 5). A similar observation was reported very recently in lymphoma cells (37). From our initial experiments, we conclude that immunotoxin treatment of DLD1 cells does not by itself result in apoptosis despite a complete reduction in protein synthesis and loss of Mcl-1. However, when ABT-737 was added in combination, there was a significant increase in caspase 3 activity, PARP cleavage, loss of Mcl-1, and cell death (Fig. 2B and D, lanes 3 and 4).

Effect of ABT-737 on other cell lines

To determine the broad utility of immunotoxin–ABT-737 combinations, we expanded our testing to include other cell lines and other immunotoxins. Combinations of ABT-737 and HB21-PE40 were added to SKOV3, an ovarian cancer cell line reported to be resistant to certain toxin-based agents (38). As noted with DLD1
cells, when immunotoxin and ABT-737 combinations were used, there was an enhancement of killing (by ~20-fold) over the addition of immunotoxin alone (Fig. 3A). Next, we tested the cervical cancer cell line KB3-1 (an immunotoxin-sensitive cell line) with immunotoxins targeted either to the transferrin receptor (Fig. 3B) or to mesothelin (Fig. 3C). Again, there was a profound increase of immunotoxin activity with an ~20-fold greater toxicity when ABT-737 was present compared with immunotoxin alone. For KB3-1 cells, we extended the analysis to look at Western blots of apoptosis-related proteins after treatments (for 24 hours) with single agents or combinations of immunotoxin and ABT-737. As shown in Fig. 3D, the presence of ABT-737 resulted in PARP cleavage, Mcl-1 degradation, and loss of pro-caspase 3. When the same treatments were extended to 48 hours, immunotoxin alone resulted in PARP and pro-caspase 3 cleavage (data not shown), indicating that KB3-1 cells were not resistant to apoptosis, but in the presence of ABT-737, cell death is evident earlier.

**ABT-737 specifically enhances toxin translocation from the ER**

Because KB3-1 exhibited no apparent resistance to immunotoxin treatment, the 20-fold enhancement of HB21-PE40 and SS1P cytotoxicity by ABT-737 was unexpected. To explore the mechanism of the ABT-737 effect, we did additional experiments using the same WST-1 in which ABT-737 was added in combination with three other agents that inhibit protein synthesis. These included native DT, cycloheximide, and HB21-CET40, a newly described immunotoxin made from a truncated exotoxin derived from *Vibrio cholerae*, which also, like PE, ends in a KDEL-like sequence (35). Only ABT-737 in combination with PE- or cholera exotoxin (CET)–immunotoxins produced a >10-fold enhancement of toxicity (Figs. 3A–C and 4C). Both these agents end in KDEL-like sequences and are reported to translocate to the cytosol from the ER (12, 14). With DT and cycloheximide, which reach the cytosol by ER-independent routes, there was no evidence of ABT-737–mediated enhancement (Fig. 4A...
and B). This result does not contradict the observation reported in Fig. 2C, in which there was an increase in caspase 3 activity with ABT-737 and cycloheximide, but reflects what each assay measures (see Discussion). The enhancement of PE cytotoxic activity could be due to the increased delivery of toxin or increased susceptibility of ABT-treated cells for ADP-ribosylated EF2. The lack of enhancement of DT cytotoxicity seemed to rule out the latter. Therefore, we sought to investigate the delivery of the enzymatic domain of PE to the cytosol. We reasoned that if PE were delivered in greater amounts, there would be a greater reduction in protein synthesis. Alternatively, if ABT-737 were acting downstream of ADP-ribosylation, then protein synthesis levels would be the same regardless of the presence of ABT-737. Results indicated that for two PE-based immunotoxins (SS1 is shown), there was a 25-fold greater reduction in the level of protein synthesis in the presence of ABT (Fig. 4D, and data with HB21-PE40; data not shown) than in its absence. This result suggests that ABT-737 could promote the delivery of a greater number of PE molecules from the ER to the cytosol.

**ABT-737 treatment produces stress within the ER**

Because the cellular uptake of immunotoxins, added at subnanomolar concentrations, is well below the detection limit for immunoblot analysis, we could not document directly that additional molecules of toxin were delivered to the cytosol. Instead, we sought evidence that ABT-737 was interacting with the ER. In various cell types, Bcl-2 family members are found associated with the ER (39, 40), and thus, the addition of ABT-737 could cause ER stress in these cells. To test if ABT-737 interacted with the ER and provoked an ER stress response, we incubated KB3-1 or DLD1 cells for 4 hours with either ABT-737 or with DTT; the latter being a well-known mediator of ER stress. Treated cell extracts were analyzed for the transcription factor ATF4 (41). Results indicate that DTT strongly upregulated the ER stress response in both cell lines, whereas ABT-737 produced a strong stress response in KB3-1 cells and a moderate one in DLD1 cells (Fig. 5A). Overall, our results are consistent with ABT-737 acting on the ER and causing, either directly or indirectly, an increased translocation of PE- or CET-based immunotoxins to the cytosol.

![Figure 3. ABT-737 enhances immunotoxin activity in SKOV3 and KB3-1 cells. A, B, and C, cells were treated as indicated for 48 h and viability was assessed using a WST-1 assay. D, KB3-1 cells were treated as indicated for 24 h, cell lysates prepared and probed with antibodies to PARP, procaspase 3, Mcl-1, and tubulin.](http://www.aacrjournals.org)
Clinical considerations for immunotoxin-ABT combination therapy

Assays for cell viability routinely involve exposing cells to toxins continuously for 48 hours. However, PE-based recombinant immunotoxins have plasma half-lives of only 2 to 7 hours (4, 7–9), and thus, might not remain in contact with target cells for more than a few hours. To simulate short-term exposures, immunotoxin–ABT-737 combinations were applied for 4 hours. Surviving cells were then visualized after 6 days (Fig. 6A and B). When the immunotoxin HB21-PE40 was used, the combination with ABT-737 resulted in a >10-fold enhancement of killing (Fig. 6A). Similarly, ABT-737 enhanced SS1P activity (Fig. 6B). To compare immunotoxin treatment with another agent that inhibits protein synthesis, a parallel experiment was conducted with cycloheximide. In contrast with the immunotoxins, a 4-hour exposure with cycloheximide and ABT-737 did not result in any significant cell killing (data not shown). Because immunotoxins act catalytically and have no known intracellular inhibitors, they will continue to be active once delivered to cytosol. In contrast, cycloheximide is a reversible inhibitor of protein synthesis and therefore its effect would only be noted if it is present continually. From the above data, we conclude that ABT-737 might be particularly useful in promoting immunotoxin killing even with short-term exposures.

Although ABT-737 itself is not destined for clinical use, its “orally available” variant, ABT-263, is currently under clinical evaluation. To confirm that ABT-263 has a similar toxin-enhancing activity as ABT-737, we did additional

Figure 4. ABT-737–mediated enhancement of KDEL-ending toxins. KB3-1 cells were incubated with various concentrations of DT (A), cycloheximide (B), or an immunotoxin made with a truncated exotoxin from V. cholerae (C) and assayed for viability 48 h posttreatment. Toxin treatments were made alone or in combination with ABT-737 at the concentrations indicated. D, the SS1P immunotoxin was added to cells either alone or in combination with ABT-737 for 18 h and then assayed for inhibition of protein synthesis.

Figure 5. ABT-737 causes ER stress. Lysates of ABT-treated DLD1 and KB3-1 cells were probed for the ER stress marker, ATF4. Lysates were prepared after 4 h of treatment with either 10 μmol/L of ABT-737 or 10 mmol/L of DTT.
cytotoxicity and caspase 3 activity assays with this compound (Fig. 6C and D). DLD1 cells were incubated with HB21-PE40 in the presence or absence of ABT-263. Our results indicate that ABT-263 has similar toxicity-enhancing activities as those noted with ABT-737 (Fig. 6C and D). ABT-263 also enhanced KB3-1 and SKOV3 killing (data not shown). We conclude that immunotoxin resistance could be overcome using ABT-263 and that future in vivo investigations could proceed with this orally available compound.

Discussion

Here, we show that the BH3-only mimetic, ABT-737, has two properties: one, it can overcome resistance to immunotoxin-mediated apoptosis; and two, it can increase immunotoxin delivery from the ER to the cytosol resulting in enhanced killing by as much as 20-fold. Both activities were achieved using concentrations (3 or 10 μmol/L) of ABT-737 that were nontoxic when added alone. Combinations of immunotoxin and ABT-737 were effective at overcoming resistance because each agent targeted a distinct Bcl-2 family member. By inhibiting protein synthesis, immunotoxin treatment promotes a decline in Mcl-1 levels. This was reported previously for both cycloheximide (27) and PE immunotoxins (26), and was confirmed here. ABT-737 is a peptide mimic modeled on a BH3-only domain and exhibits high binding affinity binding for Bcl-2, Bcl-xl, and Bcl-w (19). Binding of ABT-737 to either Bcl-2 or Bcl-xl neutralizes their prosurvival activity, allowing Bax or Bak to initiate the intrinsic arm of the apoptosis pathway. ABT-737 binds to Mcl-1 poorly and because of this, is not effective as a single agent against cancers expressing high levels of Mcl-1 (42). For these reasons, the effectiveness of immunotoxin-ABT combinations in overcoming resistance to apoptosis was not unexpected.

However, ABT-mediated enhancement of PE delivery to the cytosol was completely unexpected. In our study, this was noted in several ways: there was a 20-fold enhancement of the cytotoxic activity of two PE immunotoxins and one CET-immunotoxin made from a KDEL-ending toxin from *V. cholerae*. In addition, ABT-737 enhanced the delivery of toxin as monitored with an inhibition of protein synthesis assay. This suggested

![Figure 6](https://example.com/figure6.png)

**Figure 6.** ABT-737 and ABT-263 both exhibit immunotoxin-enhancing activity. A and B, ABT-737 enhances immunotoxin activity with even a short (4 h) exposure to the combination. ABT-737 was added in combination with immunotoxins HB21-PE40 or SS1P for 4 h, cells trypsinized and replated for 6 d. Cells that survived were visualized using methylene blue as the stain. C and D, ABT-263 enhances immunotoxin activity against DLD1 cells.
that ABT-737 was acting at a step in the toxin pathway prior to the ADP-ribosylation of EF2. Furthermore, in the same assays, there was little enhancement of DT toxicity suggesting that endosomal delivery was not involved. Because PE escapes from the ER to the cytosol, we speculated that ABT-737 was acting by altering the ER structure or function, perhaps by interacting with Bcl-2 proteins in the ER and causing a stress response. We attempted to confirm this by probing for increased ATF4 expression, a classic indicator of ER stress, and elevations of this transcription factor were noted within 4 hours of adding ABT-737 to either DLD1 or KB3-1 cells. ABT-737-mediated ER stress was not observed by Hermanson et al. (43) when analyzing several leukemic cell lines. In our unpublished results, we did not see any enhancement of PE-immunotoxin cytotoxicity when we investigated responses in the Burkitt lymphoma cell lines CA46 or Raji. Thus, ABT-737 might interact with epithelial and lymphoid-derived cell lines in different ways. ER stress is usually initiated by the release of the chaperone Bip/Gpr78 from one of three monitor transmembrane proteins, PERK, ATF6, and IRE1. Release of Bip/Gpr78 usually initiates endoplasmic reticulum-associated degradation and the retrieval of poorly folded proteins from the ER to the cytosol. We note that one theory of toxin egress from the ER is to mimic an unfolded protein and thereby act as bait for endoplasmic reticulum-associated degradation (13). By stressing the ER, ABT-737 seems to increase the efficiency of toxin transport to the cytosol. The mechanistic details of how ABT-737 interacts with the ER are currently under investigation.

In immunotoxin-resistant DLD1 cells, enhancement of caspase 3 activity was seen with both cycloheximide and HB21-PE40 in combination with ABT-737 because ABT-737 removed the block in the apoptotic pathway caused by high levels of Bcl-2 family prosurvival proteins. In this capacity, ABT-737 showed its first property of overcoming resistance to apoptosis in the presence of agents that inhibit protein synthesis. However, in immunotoxin-sensitive KB3-1 cells, when the inhibition of cell growth was measured using a WST-1 assay, there was no ABT-737–mediated enhancement of cycloheximide or DT activity. Because these cells are sensitive to protein synthesis inhibitors, the cells were killed similarly in the presence or absence of ABT-737, and no enhancement was noted because ABT-737 did not enhance the delivery of these toxins to the cell cytosol. In contrast, cells treated with combinations of HB21-CET40 and SS1P with ABT-737 were killed more efficiently and with lower doses of immunotoxin because ABT-737 not only removes the prosurvival block, but also increases the delivery of these toxins from the ER to the cytosol.

In vivo, recombinant immunotoxins are short-lived proteins with half-lives of 2 to 8 hours and might not be in contact with tumor cells very long. When we conducted a short-term assay, in which cells and agents were in mutual contact for only 4 hours, we found that the combination of immunotoxin and ABT-737 was at least 10-fold more cytotoxic than the immunotoxin alone. Because ABT-737 was removed after only 4 hours, it must act quickly to allow increased toxin egress to the cytosol. Immunotoxins are reported to be stable in the cytosol, although that is challenging to prove experimentally, our data from this experiment supports this contention.

ABT-737 is not readily available as an orally administered compound, hence, ABT-263 was developed (20). Here, we extend our initial findings to ensure that the clinically relevant compound also enhances PE-immunotoxin activity.

In summary, our major findings include the discovery that complete inhibition of protein synthesis and loss of Mcl-1 does not always lead to cell death. To overcome this resistance, we used the BH3-only mimetic, ABT-737, which implicates Bcl-2, Bcl-xl, or Bcl-w as the proximate cause of resistance. Furthermore, ABT-737 enhanced the delivery of active PE to the cytosol suggesting that prosurvival Bcl-2 proteins are located in the ER and that ABT-737 disrupts ER function via the neutralization of one or more of these proteins. Our data support the preclinical development of ABT-263–immunotoxin combinations for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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