Role of the Microenvironment for Radiosensitization by Patupilone

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Abstract

Purpose: The combined treatment modality of ionizing radiation (IR) and the clinically relevant microtubule-stabilizing compound patupilone (epothilone B, EPO906) is a promising approach for anticancer therapy. Here, we investigated the role of the tumor microenvironment for the supra-additive in vivo response in tumor xenografts derived from patupilone-sensitive and patupilone-resistant non-small cell lung cancer cells.

Experimental Design: The treatment response to a combined regimen of patupilone and IR was investigated in vitro and in tumor xenografts derived from wild-type A549 and A549.EpoB40 cells, which are resistant to patupilone due to a β-tubulin mutation.

Results: In both A549 and A549.EpoB40 cells, proliferative activity and clonogenicity were reduced in response to IR, whereas patupilone, as expected, inhibited proliferation of the mutant cell line with reduced potency. Combined treatment with patupilone and IR induced a cytotoxic effect in vitro in an additive way in A549 cells but not in the tubulin-mutated, patupilone-resistant A549.EpoB40 cells. A supra-additive tumor growth delay was induced by combined treatment in xenografts derived from A549 cells but not in xenografts derived from A549.EpoB40 cells. Histologic analysis revealed a significant decrease in tumor cell proliferation (Ki-67) and microvessel density and a treatment-dependent change of tumor hypoxia in A549 but not A549.EpoB40 xenografts.

Conclusions: Using a genetically defined patupilone-sensitive and patupilone-resistant tumor model, we here showed that the major cytotoxic effect of the combined treatment modality of IR and patupilone is directed against the tumor cell compartment. The induced antiangiogenic effect derives indirectly from the tumor cell.

Interference with microtubule function is a promising strategy for anticancer therapy (1). This approach has been extensively validated by the use of taxanes (microtubule stabilizers) for the treatment of a wide variety of human malignancies. The suppression of microtubule dynamics interferes with mitotic spindle formation, leading to cell cycle arrest in M phase and eventually to apoptosis or postmitotic cell death. The primary mechanism of microtubule-stabilizing agents (MSA) at the biochemical level is well investigated, but the signaling consequences relevant for their cytotoxic effect are far from clear. Furthermore, treatment with taxanes (paclitaxel and docetaxel) is limited by taxane-related toxicities and the development of multidrug resistance. This has prompted an ongoing worldwide search for novel microtubule-targeting compounds. One new class of MSAs are the epothilones, which are structurally distinct from the taxanes and may overcome some of their limitations, suggesting a promising new treatment approach for cancer (2–4).

Epothilones are nontaxoid MSA of bacterial origin, which share the same binding site on β-tubulin with taxanes (5, 6). One of the epothilones, patupilone (epothilone B, EPO906), is currently in phase II clinical development in several different solid tumors and is in phase III clinical trials for ovarian cancers in patients refractory to carboplatin-taxane treatment. Patupilone retains activity against P-glycoprotein-expressing multidrug-resistant cells both in vitro and in vivo (5) and shows different clinical toxicities to the taxanes (6, 7). Apart from the direct tumor-cytotoxic action, patupilone has also shown antivascular (8) and antiangiogenic (9) effects. The antiangiogenic effect might be linked to different mechanisms either directly targeting endothelial cells (10) or indirectly interfering with the secretion of proangiogenic agents from tumor cells (11, 12).

The accumulation of cells in the most radiosensitive G2-M phase of the cell cycle represents the current rationale to combine MSAs with ionizing radiation (IR) and promises a strong radiosensitizing effect in combination with IR (2, 13, 14). Our own previous in vitro and in vivo experiments done with patupilone in combination with IR showed an at least additive cytotoxic effect in various cancer cell types in vitro and strong supra-additivity of the combined treatment regimen against tumor xenografts in vivo. Interestingly, also a S-phase

patupilone and IR alone and in combination also be due to effects on the microenvironment within the solid sensitization, which is not limited to the tumor cell but may direct against the tumor cell compartment and that the induced antiangiogenic effect, which contributes to the synergistic treatment response of this combined treatment modality, derives indirectly from the tumor cell. These preclinical experiments are of relevance for clinical strategies that target the microtubule cytoskeleton and hypoxia-inducible factor dysregulation in combination with IR.

## Materials and Methods

Patupilone (epothilone B, EPO906) was provided by the Chemistry Department of Novartis Pharma. The human cell line pair A549 and A549.EpoB40 was obtained from the laboratory of Dr. Susan Band Horwitz.

**Cell cultures, drug preparation, and irradiation.** The human non-small cell lung cancer lines A549 and A549.EpoB40 were grown in RPMI 1640 containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine at 37°C in 5% CO2. A549.EpoB40 is derived from A549 by stepwise selection with patupilone because it contains a defined β-tubulin mutation (3, 11, 16, 17). In this manner, we aimed to distinguish between a tumor cell and/or tumor microenvironment-directed effect of the different treatment modalities. Our results show a major tumor cell-directed effect with a subsequent indirect antiangiogenic effect especially by the combined treatment modality of patupilone in combination with IR.

**Histology and immunohistochemistry.** Tissues were immersion fixed in 4% PBS-buffered formalin and embedded in paraffin. Sections (3 μm thick) were mounted on glass slides (SuperFrost Plus; Menzel), deparaffinized, rehydrated, and stained with H&E using standard histologic techniques. In addition, CC1 (antigen retrieval solution; Ventana Medical Systems)-pretreated sections were immunostained for Ki-67 (rabbit clone SP6; dilution 1:100; NeoMarkers), CD31 (rabbit polyclonal, ab28364; dilution 1:50; Abcam), GLUT1 (rabbit polyclonal, MYM AB 1351; dilution 1:1,000; Chemicon International) using a Discovery immunohistochemistry staining system (Ventana Medical Systems). Detection of primary antibody was done with a biotinylated anti-rabbit IgG antibody (Jackson ImmunoResearch) and the iView DAB kit (Ventana Medical Systems). Hypoxia detection, pimonidazole hydrochloride (60-80 mg/kg) was applied intravenously 45 min before sacrifice. CC1-pretreated sections were incubated with Hypoxyprobe-1 (mouse IgG1; dilution 1:300; Chemicon International) conjugated with FITC. Detection of primary antibody was done with a biotinylated mouse anti-FITC antibody (Jackson ImmunoResearch) and the iView DAB kit. All immunostains were counterstained with hematoxylin.

To determine proliferative activity, Ki-67-positive tumor cells were counted in 5 to 10 randomly chosen visual fields (magnification ×200) in each xenograft (n = 4 for each group). The mean Ki-67-positive cell count from these fields was determined. MicrovesSEL density (MVD) was determined in 5 to 10 randomly chosen visual fields (high-power fields) in each of three equally treated vital tumor tissues at ×100 magnification (0.3 mm2 visual field size). Hypoxia staining was quantified by manual counting using a grid of 1,200 points in two photographs per tumor, which displayed the whole tumor section.

**Transfections and reporter gene assay.** Both cell lines were stably transfected with a vector construct containing two copies of the Transferrin promoter hypoxia response element (HRE) and SV40 promoter in front of the firefly luciferase gene. Stably transfected, hypoxycin-selected A549 and A549.EpoB40 cell lines were tested for luciferase enzymatic activity with a commercial kit (Bright-Glo luciferase assay; Promega) using a GloMax 20/20 luminometer. Hypoxic conditions were mimicked using dimethyloxaloylglycine (BioCon), a prolly-4-hydroxylase inhibitor, which prevents hypoxia-inducible
factor (HIF) degradation at prior tested nontoxic concentrations of 0.25 and 1 mmol/L, respectively. To discriminate between treatment-induced cell death and treatment-interfered HIF signaling, cells were transiently cotransfected with a Remilla luciferase-expressing control vector. Patupilone was applied 24 h after transient transfection. Dimethylxalloylglycine treatment was started 24 h and irradiation (5 Gy) 28 h after patupilone treatment. Luciferase activity was determined 14 h after treatment with dimethylxalloylglycine.

**Statistical analysis.** Statistical analysis of the in vivo tumor growth data was done with the Mann-Whitney U test. The absolute tumor growth delay was defined as the time for tumor volume in the treated groups to triplicate the initial treatment size minus the time in the untreated control group to reach the same size. Kruskal-Wallis one-way ANOVA was used to test for significant differences of Ki-67, CD31, and hypoxia following the various treatments. Treatments effects (difference between control and treatments) were analyzed using the Mann-Whitney U test. The level of significance was set at 0.05. The calculations were all done using the StatView program (SAS Institute) version 5.0.1.

**Results**

**Antiproliferative effect of patupilone under normoxic and hypoxic conditions.** The antiproliferative effect of patupilone was tested over 72 h in the A549 wild-type and A549.EpoB40 mutant cell lines under normoxic and hypoxic conditions. A dose-dependent decrease of the proliferative activity was observed in the A549 tumor cells (IC50 ~ 0.2 nmol/L), whereas a >100-fold higher concentration (>20 nmol/L) was required to obtain the same antiproliferative effect in the mutant A549.EpoB40 cell line. No difference with regard to the antiproliferative effect by patupilone was observed under normoxia and under hypoxia (1% pO2; Fig. 1A and B).

**Antiproliferative and clonogenic cell death-inducing effect by combined treatment with patupilone and IR.** The antiproliferative effect of patupilone in combination with IR was tested using increasing concentrations of patupilone and doses of IR. In the patupilone-sensitive A549 cell line, an additive antiproliferative effect was induced with 5 Gy, a dose that shows <50% inhibition of proliferation over 72 h in response to irradiation alone (Fig. 2A and B). No radiosensitizing effect could be detected in the drug-resistant A549.EpoB40 cell line at various doses of patupilone and IR. Interestingly, these cells were also slightly more radioresistant than the wild-type counterpart cell line (Fig. 2C and D). Patupilone was also highly potent against human umbilical vein endothelial cells. Picomolar concentrations of patupilone were sufficient to inhibit endothelial cell proliferation and cell viability alone and in combination with IR (data not shown). Clonogenic survival assays were done with both cell lines and increasing concentrations of patupilone and IR. Clonogenicity was reduced in an at least additive way in the A549 cell population. In contrast, the mutant cell line A549.EpoB40 was refractory to patupilone alone up to 10 nmol/L, and patupilone did not sensitize for IR (Fig. 2E and F).

**Effect of combined treatment with patupilone and IR on growth of tumor xenografts.** We previously observed a supra-additive effect of a combined patupilone/IR treatment on tumor growth delay in xenografts derived from radiosensitive SW480 human colon adenocarcinoma cells (15). In the tumor xenografts derived from the wild-type A549 cell line, a small growth delay was induced by 3 × 1 Gy irradiation compared with untreated control tumors. Growth was significantly inhibited by patupilone (1 × 2 mg/kg) treatment alone (P = 0.022). In response to the combined treatment modality, a supra-additive tumor growth delay was observed. Tumor growth in the combined treatment group was significantly inhibited compared with IR- or patupilone-treated groups (P = 0.010 and 0.013; Fig. 3A). The absolute growth delay to triple the initial tumor volume at the start of treatment (200 mm² ≥ 10%) was most enhanced with the combined treatment modality when compared with the absolute tumor growth delay in response to patupilone or IR alone [26 days (patupilone + IR) versus 6 days (patupilone) and 2 days (IR)], respectively (data not shown). In contrast, no tumor growth delay was induced by the same dose of patupilone in tumors derived from the drug-resistant A549.EpoB40 cells, indicating that the tumor growth suppression effect of patupilone primarily derives from tumor cell-directed cytotoxicity (Fig. 3B). The tumors derived from the mutated cell line were also more radiation-resistant than the wild-type counterparts. Nevertheless, a treatment regimen of 4 × 3 Gy alone induced a significant growth delay in tumors derived from these cells (P = 0.004). On the other hand, combined treatment did not further increase the antitumor effect, and even a slightly diminished absolute tumor growth delay was observed after combined treatment versus treatment alone (1 × 2 mg/kg) (P = 0.022). In response to the combined treatment modality, a supra-additive tumor growth delay was observed. Tumor growth in the combined treatment group was significantly inhibited compared with IR- or patupilone-treated groups (P = 0.010 and 0.013; Fig. 3A). The absolute growth delay to triple the initial tumor volume at the start of treatment (200 mm² ≥ 10%) was most enhanced with the combined treatment modality when compared with the absolute tumor growth delay in response to patupilone or IR alone [26 days (patupilone + IR) versus 6 days (patupilone) and 2 days (IR)], respectively (data not shown). In contrast, no tumor growth delay was induced by the same dose of patupilone in tumors derived from the drug-resistant A549.EpoB40 cells, indicating that the tumor growth suppression effect of patupilone primarily derives from tumor cell-directed cytotoxicity (Fig. 3B). The tumors derived from the mutated cell line were also more radiation-resistant than the wild-type counterparts. Nevertheless, a treatment regimen of 4 × 3 Gy alone induced a significant growth delay in tumors derived from these cells (P = 0.004). On the other hand, combined treatment did not further increase the antitumor effect, and even a slightly diminished absolute tumor growth delay was observed after combined treatment versus treatment
with IR alone [14 days (IR) versus 9 days (patupilone + IR); data not shown]. These results indicate that the major antitumor effect of the investigated treatment modality derives from tumor cell-directed cytotoxicity. A direct antiangiogenic effect of patupilone alone or in combination with irradiation is unlikely to contribute to the observed tumor growth delay. Determination of body weight changes only revealed a minor patupilone-dependent transient weight loss over 48 h after treatment.

Fig. 2. Antiproliferative effect of patupilone alone and in combination with IR (5 Gy). Lung adenocarcinoma cells A549 (A and B) and mutant cells A549:EpoB40 (C and D) were exposed to increasing concentrations of patupilone and IR. Clonogenic survival after treatment with patupilone and IR was determined for A549 cells (E) and mutant A549:EpoB40 cells (F) treated with patupilone 18 h before irradiation.
reduced by 32% 84 h after treatment with patupilone alone, whereas irradiation and the combined treatment reduced the proliferative activity by 54% and 71%, respectively ($P < 0.0001$). No change in the proliferative activity could be detected in A549.EpoB40-derived tumor sections after patupilone treatment. Irradiation reduced the proliferative activity by 22% ($P < 0.0001$), without any further effect in the combined treatment group (Fig. 4A and B). To examine the effect of the different treatment modalities on the tumor vasculature, MVD was determined by CD31 immunohistochemistry. Patupilone reduced the MVD in the drug-sensitive tumors by 25% at the 84 h time point when compared with control tumors ($P = 0.0003$). Irradiation and combined treatment reduced the MVD by 37% and 47%, respectively ($P < 0.0001$). In the drug-resistant tumors, a tendency in MVD reduction was only observed in the irradiated group ($P = 0.074$; Fig. 4C and D).

Treatment-induced changes of the tumor vasculature may also affect the pO₂ in the tumor tissue. We therefore determined treatment-dependent changes of tumor oxygenation by injection of the hypoxia probe pimonidazole, which specifically accumulates in hypoxic tissue areas. Treatment-dependent changes of hypoxic areas were determined in histologic sections 84 h after treatment. A significant increase of pimonidazole accumulation was determined in A549-derived tumors after treatment with patupilone ($P = 0.0019$), whereas the amount of hypoxic, pimonidazole-positive areas was reduced in the irradiated group ($P = 0.0045$). Interestingly, the amount of hypoxic areas was similar to untreated tumors after treatment with patupilone in combination with IR ($P = 0.747$). No change of pimonidazole accumulation was observed in any of the treatment groups in A549.EpoB40-derived tumor sections (Fig. 4E and F). These results indicate that changes in the tumor microenvironment in response to the different treatment modalities mainly derive from the treatment effect directed against the tumor cells (see below).

**Treatment-interfered expression of hypoxia-induced gene expression.** The HIF represents the major determinant in tumor cells for hypoxia-regulated expression of proangiogenic factors and patupilone reduced the level of the hypoxia-sensitive HIF-1α subunit under hypoxic conditions in the patupilone-sensitive A549 cell line as determined by Western blotting (data not shown). Using a reporter gene assay where luciferase expression is under the control of multiple HREs, the treatment response of patupilone and IR alone and in combination was quantitatively assessed in the patupilone-sensitive and patupilone-resistant cell lines. To normalize for transfection efficacy and cell numbers, cells were cotransfected with the Renilla luciferase-expressing vector system. To mimic hypoxic conditions, cells were incubated in dimethyloxaloylglycine, thereby up-regulating the endogenous HIF level and inducing luciferase expression 8.5-fold over control conditions (data not shown). Treatment of the patupilone-sensitive A549 cells with increasing doses of the MSA significantly reduced luciferase expression already at subnanomolar concentrations ($P = 0.010$). Of note, the A549.EpoB40 cells were resistant up to low nanomolar concentrations of patupilone (20 nmol/L), indicating a role of microtubule interference in HIF-1α-regulated gene expression (Fig. 5A). Treatment of cells with increasing doses of irradiation only minimally affected luciferase expression (data not shown). Treatment with increasing doses of patupilone in combination with IR (5 Gy) additionally
decreased luciferase expression ($P = 0.018$) in the A549 cell line, indicating an increased stress response on the level of hypoxia-regulated gene expression (Fig. 5B).

**Discussion**

Here, we investigated the treatment response of tumor xenografts derived from MSA-sensitive and MSA-resistant lung adenocarcinoma cell lines to treatment with patupilone and IR alone or in combination. We previously identified a strong treatment response to this combined treatment modality *in vivo* in the SW480 human colon xenograft model, which strongly exceeded the additive cytotoxic effect of the treatment combination *in vitro* (15). By means of this genetically defined tumor model, we now were able to differentiate between a direct tumor cell-directed and a microenvironment-directed response.

![Diagram](https://www.aacrjournals.org/content/cancer/15/4/1351/F15.large.jpg)

**Fig. 4.** Tumor cell proliferation, MVD, and changes in tumor hypoxia in response to treatment. Mice with A549-derived (A, C, and E) and A549.EpoB40-derived (B, D, and F) xenografts were treated with vehicle, patupilone (2 mg/kg), IR (3 × 1 and 3 × 3 Gy, respectively), or a combination of patupilone and IR. Mice were sacrificed 84 h after treatment and tumors were harvested, formalin fixed, and stained for the Ki-67 (A and B), CD31 as marker of tumor cell proliferation and MVD (C and D), and antibodies against the exogenous hypoxia marker pimonidazole hydrochloride for the immunohistochemical detection of tumor hypoxia (E and F), respectively. Mean ± SE value per group. *, $P < 0.01$; **, $P < 0.001$. 

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![Image](https://www.aacrjournals.org/content/cancer/15/4/1351/F15.large.jpg)
cotoxic in vivo effect in response to the different treatment modalities.

We observed a cytotoxic and tumor growth delay effect in the tumors derived from the MSA-sensitive adenocarcinoma cell line A549 in response to irradiation and patupilone alone and a supra-additive response to combined treatment. As expected, patupilone reduced the proliferative activity of the MSA-resistant cell line A549.EpoB40 in vitro only at >100-fold the concentration necessary for the wild-type cell line (3, 18). As determined here in this report, no tumor growth delay could be observed in vivo with treatment of patupilone alone and no enhancement of an IR effect was induced on combined treatment in tumors derived from the MSA-resistant cell line. Of note, patupilone induced an antiangiogenic response with reduced MVD and enhanced hypoxia in the wild-type but not MSA-resistant tumors.

The wild-type A549 cell line was also more sensitive to treatment with increasing doses of IR than A549.EpoB40 cells, which carry a specific Gln-to-Glu mutation at residue 292 in the M loop of β-tubulin and which changes the affinity of β-tubulin to the MSA (3). We cannot exclude that additional mutations are present in the patupilone-resistant cells, but most likely the known β-tubulin mutation not only reduces the affinity to patupilone but also affects microtubule dynamics, which may subsequently render the cells more radiation resistant. Importantly, the proliferative activity and growth kinetics of untreated cells or tumors derived from the two different cell lines were similar. An increased radiation resistance was present in the patupilone-resistant tumor cells and not in the sensitive ones and thus still enabled to investigate the involvement of the tumor microenvironment in response to the combined treatment modality of IR with patupilone.

The potent antiangiogenic activity of MSAs was proposed to be a major mechanism leading to the successful anticaner activity of this class of compounds (9, 11, 19, 20). Patupilone has an antiproliferative and apoptosis-inducing effect in microvascular and macrovascular endothelial cells (9) and our own in vitro experiments done with primary human umbilical vein endothelial cells confirmed the high potency of patupilone against this cell type (data not shown). Patupilone also induces vascular disruption leading to reduced tumor blood volume (8). We showed previously an at least additive antitumor effect by the combined treatment of IR with different inhibitors of angiogenesis, such as the VEGF receptor tyrosine kinase inhibitor PTK787 or the dual receptor tyrosine kinase inhibitor AEE788 (21, 22). Based on the putative direct antiangiogenic property of patupilone, a partial tumor growth delay effect was expected on treatment with patupilone alone or in combination with IR even in tumors derived from the patupilone-resistant tumor cells (A549.Epo40). However, treatment with neither patupilone alone nor in combination with IR reduced tumor growth or increased tumor growth delay, respectively, in A549.Epo40-derived tumors.

A strong reduction in MVD and change in tumor hypoxia on treatment with patupilone alone was only detected in tumors derived from the A549 wild-type cells and not in tumors derived from the patupilone-resistant tumor cells. Despite direct in vitro cytotoxicity against endothelial cells (9), our results indicate that the antiangiogenic effect of patupilone in vivo is indirectly induced by interference on the level of the tumor cellular stress response. Our in vitro experiments done with the hypoxia-regulated reporter gene assay clearly showed that patupilone reduces the expression of the HIF-1 “transcriptome” such as VEGF and other genes involved in angiogenesis and hypoxic adaptation but only in patupilone-sensitive tumor cells, thereby leading to an indirect tumor cell-mediated antiangiogenic effect of patupilone. Patupilone might also directly target the microtubule system in the tumor endothelial cell compartment but might be less cytotoxic in presence of sufficient VEGF and other survival factors secreted from the tumor cells. The radiosensitivity of the tumor vascular network codetermines the tumor response to IR (23). Therefore, pharmacologic approaches, which interfere with the survival signaling of endothelial cells, render these cells more radiosensitive and subsequently enhance the tumor response to IR.
A major obstacle for the successful tumor response to IR is tumor hypoxia, which renders tumor cells two or three times more radioresistant than under normoxic conditions. Of note, our in vitro experiments revealed that patupilone is as potent against tumor cells under normoxic as under hypoxic conditions. Thus, a combined treatment modality of patupilone in combination with IR also bears promise against tumors with an increased hypoxic fraction. Here, we observed an increase of tumor hypoxia induced by patupilone alone presumably through this antiangiogenic, indirect MVD-reducing effect. We investigated previously the dynamics of tumor hypoxia in response to inhibitors of angiogenesis alone and in combination with IR and observed that irradiation counteracts the risk of treatment-induced hypoxia by the inhibition of angiogenesis (21, 22). Although treatment with the VEGF receptor/tyrosine kinase inhibitor PTK787/ZK222584 also increased overall and local tumor hypoxia, combined treatment with IR resulted in extended tumor growth delay and tumor cell apoptosis but no increase in tumor hypoxia (22). As the radiosensitivity of the tumor vascular network codetermines the tumor response to IR (23), pharmacologic approaches, which interfere with the survival signaling of endothelial cells, render these cells more radiosensitive and subsequently enhance the tumor response to IR. The reduction of survival factors secreted by the tumor cells, after treatment with patupilone, leaves the endothelial cells more vulnerable to direct irradiation. Nevertheless, combined treatment of patupilone with radiation did not enhance tumor hypoxia relative to untreated tumors as if radiation antagonizes the patupilone-induced increase of tumor hypoxia.

The mechanisms underlying this antagonistic effect is unclear, but it is conceivable that enhanced cell death together with reduced proliferation of tumor cells may reduce the intratumoral oxygen demand to a level that can still be met by the damaged tumor vasculature and thereby avoid a hypoxic state in the allograft (24, 25). This mechanism might be relevant for the combined treatment with direct and indirect inhibitors of angiogenesis.

In summary, our results using in vivo tumor models derived from patupilone-sensitive and patupilone-resistant variants of the non-small cell lung cancer cell line A549 strongly suggest that the major antitumor effect of patupilone and a combined patupilone/IR treatment is mainly due to directed effects on the tumor cell compartment and that patupilone may induce an antiangiogenic effect in an indirect way through tumor cell targeting. Overall, these preclinical experiments are of strong relevance for a clinical strategy and further support the promising treatment modality of patupilone alone and in combination with IR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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