Modulation of the Tumor Microvasculature by Phosphoinositide-3 Kinase Inhibition Increases Doxorubicin Delivery In Vivo

Naseer Qayum1, Jaehong Im1, Michael R. Stratford1, Eric J. Bernhard2, W. Gillies McKenna1, and Ruth J. Muschel1

Abstract

Purpose: Because effective drug delivery is often limited by inadequate vasculature within the tumor, the ability to modulate the tumor microenvironment is one strategy that may achieve better drug distribution. We have previously shown that treatment of mice bearing tumors with phosphoinositide-3 kinase (PI3K) inhibitors alters vascular structure in a manner analogous to vascular normalization and results in increased perfusion of the tumor. On the basis of that result, we asked whether inhibition of PI3K would improve chemotherapy delivery.

Experimental Design: Mice with xenografts using the cell line SQ20B bearing a hypoxia marker or MMTV-neu transgenic mice with spontaneous breast tumors were treated with the class I PI3K inhibitor GDC-0941. The tumor vasculature was evaluated by Doppler ultrasound, and histology. The delivery of doxorubicin was assessed using whole animal fluorescence, distribution on histologic sections, high-performance liquid chromatography on tumor lysates, and tumor growth delay.

Results: Treatment with GDC-0941 led to approximately three-fold increases in perfusion, substantially reduced hypoxia and vascular normalization by histology. Significantly increased amounts of doxorubicin were delivered to the tumors correlating with synergistic tumor growth delay. The GDC-0941 itself had no effect on tumor growth.

Conclusion: Inhibition of PI3K led to vascular normalization and improved delivery of a chemotherapeutic agent. This study highlights the importance of the microvascular effects of some novel oncogenic signaling inhibitors and the need to take those changes into account in the design of clinical trials many of which use combinations of chemotherapeutic agents. Clin Cancer Res; 18(1); 161–9. ©2011 AACR.

Introduction

The induction of angiogenesis is essential for the supply of oxygen and nutrients during tumor growth. However, compared with normal tissues, tumor vasculature is disorganized with unstructured morphology and poor function. Vessels are tortuous and dilated, lack pericyte coverage, and exhibit an abnormal basement membrane (1–3). This results in a heterogeneous, inconsistent and often inadequate blood supply to the parenchyma. In addition, tumor vasculature tends to be more permeable than normal vessels resulting in a corresponding increase in interstitial pressure (4). All these factors contribute to poor perfusion in tumors and promote tumor hypoxia as a consequence.

Cytotoxic drug delivery is impeded because of poor perfusion as well. Tumor hypoxia itself may also impede the action of some drugs and induce resistance mechanisms (5, 6). Radiation therapy is also adversely affected by hypoxia, as oxygen is required for the maximal fixation of the free radical species that cause DNA damage (7). Cytotoxic therapies are thus adversely affected by the presence of tumor hypoxia in addition to failure of delivery because of poor perfusion and increased interstitial pressure.

Current strategies against the tumor vasculature mainly target tumor blood flow or angiogenesis. Both run the risk of adversely reducing perfusion and increasing tumor hypoxia. Drugs such as combretastatin target the tumor vessel endothelium, resulting in extensive secondary cell death in the centre of the tumor. This results in increased central hypoxia (8) with a remaining rim of tumor remaining viable with the potential of regrowth, due to the presence of peripheral vascular networks and neoangiogenesis (6). Furthermore, the central necrosis can decrease the efficacy of further
Translational Relevance

Inhibitors of phosphoinositide-3 kinase (PI3K) and its signaling pathways are in development for use in cancer therapy. We have previously shown that inhibition of the RAS-PI3K-AKT signaling pathway in mice resulted in vascular normalization in their tumors leading to the hypothesis that the resulting increased perfusion would result in improved chemotherapy delivery. Here we show that this is the case of using a PI3K inhibitor. These results suggest that clinical trials incorporating PI3K inhibition should be designed to take vascular normalization and the potential benefit in drug delivery into account. They also suggest that the efficacy of PI3K inhibition might be monitored in vivo using imaging to detect such markers as increased perfusion or decreased hypoxia as indicators of vascular changes.

cytotoxic approaches to therapy. Similarly, prolonged treatment with antiangiogenic drugs, by decreasing the expansion of vascular networks, also result in decreased perfusion and increased hypoxia.

In contrast, some antiangiogenic therapies have been shown to paradoxically enhance vascular function (9). The balance between pro- and antiangiogenic signaling is tightly controlled in normal tissues, the result of which is a regular, structured vascular network, supported by a basement membrane, and enclosed with pericytes. Tumor angiogenesis, however, is less well regulated and is characterized by the overproduction of proangiogenic factors, in particular VEGF (10). Targeting VEGF receptors using antibodies results in a temporary improvement in vascular function, termed "vascular normalization," and is associated with a reduction in tumor hypoxia, and increased radiation sensitivity (11, 12). However, the transient nature of this effect makes it difficult to translate into clinical practice.

Overexpression of VEGF is linked to oncogenic signaling (13). VEGF induction can be triggered by RAS signaling through the phosphoinositide-3 kinase (PI3K) pathway (14, 15). Signal transduction arising from PI3K in particular plays a major role in cancer cell signaling. PTEN, which encodes the opposing phosphatase to PI3K, is the second most commonly affected tumor suppressor gene after p53, and PIK3CA, which encodes the p110α catalytic subunit for class 1 PI3K, is also commonly mutated in cancer (16). Thus, novel PI3K inhibitors such as GDC-0941 that act on class I PI3K (17) have been developed to target this pathway, and have entered phase I trials.

Targeting this pathway may then be a viable strategy to modulate tumor vasculature as VEGF levels can be controlled through PI3K signaling and because aspects of vascular remodeling are controlled by endothelial PI3K. Indeed, we have recently shown that inhibition of EGFR-Ras-PI3K-Akt pathway at each point results in morphologic changes in tumor vasculature that improved perfusion and resulted in a sustained reduction in tumor hypoxia (18). In that study, treatment of mice bearing SQ20B tumors with a class 1 PI3K inhibitor, PI-103 led to decreased VEGF expression accompanied by vascular changes including increased vascular area, increased vascular length, and decreased tortuosity. These vascular modifications were persistent and led to decreased tumor hypoxia and increased tumor perfusion. This result led us to hypothesize that delivery of chemotherapeutic agents could be enhanced by the vascular changes resulting from inhibition of PI3K.

Although the efficacy of PI3K inhibitors as monotherapeutic agents is important, it is also of vital interest in evaluating how best they would be incorporated into current cytotoxic therapy regimes as part of combination therapy, which is the current mainstay of clinical practice. Here we examine the effect of pretreatment with the PI3K inhibitor GDC-0941 on vascular structure and on the consequent effects on doxorubicin delivery.

Materials and Methods

Cell lines

SQ20B-luc cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS (HyClone) at 37°C in water-saturated 5% CO2. pGL-HRE, a luciferase reporter construct driven by 3 hypoxia-responsive element (HRE) binding sequences for hypoxia-inducible factor (HIF) kindly provided by Christopher Pugh (Oxford) had been introduced into SQ20B and luciferase had been shown to be hypoxia responsive (18).

Inhibitor treatment of mice

All animal experiments were carried out in accordance with U.K. Home Office regulations and the United Kingdom Coordinating Committee on Cancer Research’s “Guidelines for the Welfare of Animals in Experimental Neoplasia” (http://www.ncrr.org.uk/csg/animal_guides_txt.pdf).

Female nu/nu mice (Charles River) were inoculated with 106 SQ20B-luc cells on the hind leg s.c. Female transgenic mice [FVB/N-Tg-MMTV-neu, 202 Mul/J] were observed and mammary tumors were assessed for size. In all experiments, animals were treated with either carrier or 12.5 mg/kg GDC-0941 by daily gavage for up to 3 days.

In vivo imaging

Optical and ultrasound/power Doppler were carried out under anesthesia (2% isofluorane in oxygen). For the detection of hypoxia in vivo, luciferin (150 mg/kg, Xenogen) was given i.p. 5 minutes before optical imaging on an IVIS 200 system (Xenogen) using a Diffuse Luminescence Imaging Tomography (DLIT) algorithm (19). Ultrasound imaging was done on a Vevo770 (Visualsonics). Doppler image analysis was done with Visualsonics software as described in reference (20). For the detection of doxorubicin in vivo, doxorubicin (30 mg/kg) was injected...
Epifluorescence imaging

Doxorubicin has autofluorescent properties that enable the evaluation of drug distribution characteristics by fluorescence-based imaging. We used a method adapted from Primeau and colleagues (21). Briefly, 30 mg/kg doxorubicin was injected 180 minutes i.v. prior to sacrifice. Vascular perfusion was visualized with anti-CD31-RPE conjugated antibody, injected 10 minutes prior to sacrifice. Tumors were immediately excised, snap frozen, sectioned, and visualized unfixed in one sitting using a Leica DM IRBE microscope with a Hamamatsu C4742-95 camera. Image analysis was done using ImageJ software (http://rsbweb.nih.gov/ij). Calculation of vessel geometry and statistics was carried out using the Trace three-dimensional software (22). Determinations of tortuosity and vessel length were as described by Norrby and colleagues (23).

Immunohistochemistry

Staining for pAkt (1:50, 3787, Cell Signaling) was detected using the ImmPress Anti-Rabbit IgG (peroxidase) Kit (Vector Laboratories), counterstained with Hematoxylin QS (Vector Laboratories), and visualized using an Axioskop microscope (Zeiss).

Method for analysis of doxorubicin in plasma and tissues

Pharmacodynamics of doxorubicin was measured by collection of plasma at 15, 90, and 180 minutes after i.p. injection of 8 mg/kg doxorubicin into mice bearing SQ20B tumors of approximately 300 mm³. The extraction method was based on that of Al-Abd and colleagues (24). Tissues were homogenized in 9 volumes 20 mmol/L KH₂PO₄ and
250 µL homogenate was incubated at 37°C for 15 minutes. 250 µL acetone and 100 µL saturated ZnSO₄ was added, mixed, and then incubated at 37°C for a further 15 minutes. Samples were mixed again, then centrifuged at 20,000 × g for 2 minutes. The upper layer was transferred to a glass vial and dried in a centrifugal evaporator. The dried extracts were reconstituted in 200 µL water, and analyzed by high-performance liquid chromatography (HPLC).

HPLC was carried out on a Waters 2695 system using a 4 µm Synergi Fusion column, 250 × 3 mm (Phenomenex) maintained at 35°C. The eluents were 5 mmol/L heptane sulfonic acid, pH 4 (acetic acid), and methanol, with a gradient from 30% to 80% methanol over 10 minutes, followed by a step up to 100% methanol over 2 minutes, held for a further 2 minutes before returning to the start conditions. The flow rate was 0.6 mL/min. Detection was by fluorescence (Waters 474) with λex 480 nm, λem 550 nm, and doxorubicin eluted at 11.7 minutes.

**Tumor growth delay**

Nu/nu mice (Charles River) were inoculated with 10⁶ cells on the hind leg s.c, and animals were randomized into treatment groups after tumors reached a volume of approximately 100 mm³. Nu/nu mice bearing SQ20B-luc xenografts of approximately 100 mm³ were treated with either control or 12.5 mg/kg GDC-0941 for 3 days (n = 10). Mice were then given either 8 mg/kg of doxorubicin as a single dose i.p., or given a control treatment as a single dose (n = 5).

**Statistical analysis**

Prism 4.0 Software (GraphPad Software) was used for statistical analysis and graph generation. Unless otherwise
stated error bars indicate SE, and \( P \) values of less than 0.05 after a 2-tailed \( t \) test are denoted by an asterisk in the figures.

To assess tumor growth delay, a comparison of the time taken to reach a target volume between all groups was made using ANOVA. Post hoc testing using the Bonferroni multiple comparison test was used to compare treatment groups. A significance level of 0.05 was considered statistically significant. Testing for synergy was done as described by Yokoyama and colleagues (25). Briefly, the fractional tumor volume (FTV) relative to treatment controls was calculated. Expected values for combination treatment were derived by multiplying the mean FTV value for GDC-0941 with that derived for doxorubicin. These were then compared with those achieved experimentally as a ratio. A ratio of more than 1 indicates a synergistic effect, and a ratio of less than 1 indicates a less than additive effect.

Results

Effect of PI3K inhibition on hypoxia and tumor blood flow in xenograft and transgenic tumor models

The SQ20B cell line overexpresses epidermal growth factor receptor (EGFR), which causes constitutive activation of RAS that in turn directs the activation of PI3K and AKT (26). The inhibitor GDC-0941 blocks class I PI3K activity (17). We used SQ20B bearing a HIF monitor of hypoxia, the hypoxia-inducible promoter composed of 3 HRE sequences linked to luciferase. We previously showed that expression of luciferase in tumors derived from this cell lines correlated with increased hypoxia in the tumor by immunohistochemistry using the nitroimidazole hypoxia marker EF5 (27, 28).

Treatment of mice with GDC-0941 bearing the SQ20B-luc tumors was initiated after a preliminary scan for luciferase expression. Three days later, the control tumors had increased luciferase expression, consistent with increased HIF expression and hypoxia. Tumors treated with GDC-0941, however, showed lower levels of luciferase expression, consistent with decreased HIF and hypoxia after a 3-day period (Fig. 1A). Immunohistochemistry confirmed PI3K inhibition with greatly diminished pAKT amounts (Supplementary Fig. S1). The tumor volumes in both treatment groups were equivalent at both times (Supplementary Fig. S2). Tumor expression of HIF was therefore significantly decreased after 3 days of PI3K inhibition consistent with our previous observation of decreased hypoxia after PI3K inhibition with PI-103.

We used 3-dimensional (3D) ultrasound power Doppler to evaluate whether changes in tumor blood flow could account for the observed reduction in tumor hypoxia. 3D representations of serial 2D power Doppler scans showed an increase in vascular flow in xenografts treated with GDC-0941 as compared with controls (Fig. 1B). Hence the reduction in tumor hypoxia was associated with an increase of blood flow. Similar results were obtained in spontaneous tumors.

Figure 3. PI3K inhibition in a transgenic MMTV-neu model increases doxorubicin distribution in vivo. Tumor-bearing FVB-MMTV-neu mice were pretreated for 3 days with vehicle or 12.5 mg/kg GDC-0941. They were then injected with 30 mg/kg doxorubicin i.v. and imaged accordingly. A, ten minutes before sacrifice, animals were injected with CD31-PE, harvested, and imaged under immunofluorescence—representative images of control and tumors treated are shown, with quantitation showing an increase in doxorubicin fluorescence after treatment with GDC-0941 (% area autofluorescence). Green, doxorubicin autofluorescence; red, vascular endothelial marker (CD31-PE). B, further quantitative analysis of vascular structure shows a decrease in vessel tortuosity, and increased vessel length after treatment with GDC-0941 as compared with controls. C, concentration of doxorubicin determined by HPLC after tumor harvest (3 hours post i.v. injection of 30 mg/kg doxorubicin) was significantly increased in tumors pretreated with GDC-0941 (\( P = 0.0055 \)).
mouse mammary tumors in the transgenic FVB-MMTV-neu mice (Fig. 1C). This model also shows enhanced signaling through PI3K (29, 30).

**Inhibition of PI3K increases delivery of doxorubicin in vivo in xenograft and transgenic tumor models**

Doxorubicin is autofluorescent, so we used in vivo fluorescence and ex vivo fluorescence imaging to evaluate the distribution of doxorubicin and its relationship to the tumor vasculature. Animals received a dose of doxorubicin (30 mg/kg) before imaging. We used a high dose of doxorubicin primarily to facilitate the detection and quantitation of doxorubicin autofluorescence based on studies by Primaux and colleagues (21). The extent of fluorescence detected gives an indication of the extent of uptake of doxorubicin in the tumor noting that some inactive metabolites will also be fluorescent. In SQ20B xenografts, we observed an increase in doxorubicin uptake in xenografts pretreated with GDC-0941 for 3 days compared with controls using whole animal fluorescence (Fig. 2A). In addition, subsequent ex vivo frozen sections showed a significant increase of doxorubicin distribution in the tumors, as determined by surface area autofluorescence after PI3K inhibition (Fig. 2B). The doxorubicin was predominantly in proximity to vessels. Decreased vessel tortuosity and increased vessel length was also observed in the treated mice (Fig. 2C), consistent with the changes in vascular flow described above. Finally, HPLC analysis confirmed a significant increase in doxorubicin concentration within the tumors from treated mice compared with controls (Fig. 2D). Treatment with GDC-0941 did not alter the pharmacodynamics of doxorubicin (Supplementary Fig. S3). We observed similar changes in vascular structure and doxorubicin distribution in the tumor after 3 days of GDC-0941 treatment in the FVB-MMTV-neu tumor model (Fig. 3).

**Combined PI3K/doxorubicin treatment results in synergistic tumor growth delay as compared with monotherapy**

We conducted a tumor growth delay assay, to evaluate whether the increased levels of doxorubicin distribution seen after treatment with GDC-0941 translated to enhanced tumor control. Nu/nu mice bearing SQ20B-luc xenografts of approximately 100 mm³ were treated with either vehicle or 12.5 mg/kg GDC-0941 daily for 3 days (n = 10). Mice were then given either 8 mg/kg of doxorubicin as a single dose i.p. or given a control treatment (n = 5). B, tumor growth delay curve for all 4 treatments. The black arrow denotes the timing of first set of treatments (GDC or GDC carrier); gray arrow denotes the second set of treatments (doxorubicin or doxorubicin carrier), as in (A). C, the time taken for a tumor to grow to 10 times its original volume (TGTV10) was plotted for each treatment group. A significant increase in tumor growth delay (**, P < 0.001) was seen between the control and the combined GDC-0941/doxorubicin group, and between doxorubicin and combined GDC-0941/doxorubicin treatments (**, P = 0.0031). Control treatments versus doxorubicin alone were also significant (***, P = 0.0026). Tumor growth delay between control versus doxorubicin alone was insignificant (P = 0.0955, not shown on graph).

The difference between the control treatments and those treated with doxorubicin alone was significant (**, P = 0.0026), confirming the cytotoxic effect of doxorubicin on our tumor model. A significant and more substantial increase in tumor growth delay (***, P < 0.001) was measured after doxorubicin administration to the group pretreated with GDC-0941. Tumor growth delay resulting from 12.5 mg/kg GDC-0941 alone compared with the control group was not significant (P = 0.0955). Further analysis using ANOVA revealed the changes between other groups were significant (P < 0.01). Comparison between groups using post hoc testing (Table 1) showed significant (P < 0.05) changes between control versus doxorubicin treatments, and the control versus combined doxorubicin/GDC-0941 treatments. Furthermore, there were significant changes between GDC-0941, versus doxorubicin/GDC-0941 and doxorubicin versus doxorubicin/GDC-0941. Finally, the FTG relative to treatment controls were calculated to assess
for synergy (Table 2). Expected values for combination treatment were derived by multiplying the mean FTV value for GDC-0941 with that derived for doxorubicin. These were then compared with those achieved experimentally as a ratio. A ratio of more than 1 indicates a synergistic effect, and a ratio of less than 1 indicates a less than additive effect. We observed a ratio of 1.76. This established that pretreatment of tumors with GDC-0941 synergistically increased tumor growth delay resulting with doxorubicin.

**Discussion**

In this report, we showed that inhibition of PI3K using GDC-0941 before administration of doxorubicin improves its distribution in the tumor. We further show that synergic tumor growth delay is observed with combination treatments compared with monotherapy. We suggest that these findings will be of vital importance when evaluating future chemotherapeutic strategies.

Modulation of the vascular component of the tumor microenvironment has been postulated as a means for improving drug delivery to solid tumors, and has mainly been approached by targeting the tumor vasculature (11). In these studies, targeting VEGF receptors normalized the tumor vasculature, resulting in a transient improvement in vascular function and a reduction in tumor hypoxia—the temporary nature of its effect making it difficult to deploy in the clinical setting. We and others (18, 29) have recently suggested that by targeting oncogenic signaling the tumor vasculature can be modulated to achieve similar effects with the additional advantage of sensitizing tumor cells themselves to cytotoxic therapy (30). We also show that these changes persist during treatment and thus appear to be more durable than VEGFR inhibition (18). Inhibition of VEGF production by tumor cells due to PI3K inhibition occurs after treatment of mice bearing tumors with EGFR inhibitors or with PI3K inhibitors and is one possible mechanism underlying the vascular alterations seen here. In addition, PI3K is known to be required for endothelial sprouting so that its inhibition might explain the decreased branching and tortuosity in the vascular structure seen after its inhibition (31). Whether treatment of a tumor with PI3K inhibition results in decreased tumor growth appears to be dependent upon the spectrum of genetic alterations leading to the signaling alterations (32, 33). Inhibition of pAKT by GDC-0941 did not lead to growth inhibition of MDA-MB-231 cells despite inhibition of this pathway while other tumors with mutation in the PI3K are growth inhibited. Thus it was not surprising that treatments of tumors leading to inhibition of signaling did not lead to growth inhibition. It is also possible that potential growth stimulation because of enhanced perfusion might be counteracted by antiproliferation signals due to the drug.

The only previous preclinical study that shows oncogenic inhibition as a strategy for improved chemotherapy delivery via changes in the vasculature are those of Cerniglia and colleagues (29), that examined targeting of the EGFR receptor. They showed an increase in tumor growth delay after

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**Table 1. ANOVA post hoc testing between control and treatment groups**

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<th>Bonferroni multiple comparison test</th>
<th>Mean difference</th>
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**NOTE:** FTV = (mean tumor volume experimental)/(mean tumor volume control).

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**Table 2. Combination therapy with GDC-0941 and doxorubicin**

<table>
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<th>Combination treatment</th>
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<th>Observed</th>
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<td></td>
<td></td>
<td>0.39</td>
<td>0.22</td>
<td>1.76</td>
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**NOTE:** FTV = (mean tumor volume experimental)/(mean tumor volume control).

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improved cisplatin delivery in the tumor parenchyma. We used a different approach targeting further downstream at the level of PI3K. By taking advantage of the autofluorescent nature of doxorubicin, we showed that the doxorubicin diffusion was directly linked to the vascular changes that occur after PI3K inhibition, in both xenograft and transgenic tumor models, and that this strategy could significantly delay tumor growth. Alterations in the pharmacodynamics of doxorubicin were not detected. We also showed the vascular normalization effect in spontaneous breast tumors triggered by the transgene MMTV-neu.

Although preclinical studies have shown great promise in the potential of combining oncogenic signal inhibitors and cytotoxic therapies, translation has proved difficult. In particular there have been various trials targeting EGFR that have shown little or no improvement (34) in outcome with the exception of Vermorken and colleagues who found improved overall survival after combining cetuximab with platinum-fluorouracil in head and neck squamous cell carcinoma (HNSCC; ref. 35). There may be a number of reasons for this. Many of these trials were designed before the tumor microenvironment effects of oncogenic inhibition were known. Indeed some of the doses used may cause antiangiogenic effects (36) which would be undesirable if enhanced chemotherapy delivery is required. Thus, scheduling of the therapeutic components is crucial, and both strategies targeting angiogenesis and oncogenic signaling have shown that modulating the tumor microenvironment before cytotoxic therapy is optimal (12, 29). Moreover, both intrinsic and acquired resistance to EGFR inhibitors is now well recognized, and targeting further downstream than the EGFR itself may be more fruitful as one of the EGFR resistance pathways involves the PI3K/Akt pathway (37). As we have previously shown that similar tumor microenvironment effects are observed at targeting either EGFR or PI3K (18), targeting further downstream in some instances may be more appropriate.

In conclusion, our studies provide evidence that inhibition of PI3K may improve chemotherapy delivery and enhance tumor growth delay. The tumor microenvironment effects of PI3K inhibition should be considered when combined these inhibitors with other chemotherapeutic drugs in future clinical trials. Radiologic techniques available in routine clinical practice such as DCE-MRI (38) and PET (39) imaging should provide the tools to monitor such future trials to maximize benefit from combined therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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