Mannan-modified Ad5-PTEN treatment combined with docetaxel improves the therapeutic effect in H22 tumor-bearing mice

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Background: It has been reported that the tumor suppressor gene, PTEN, which is inactivated in many malignant tumors, plays an important role in apoptosis, cell cycle arrest, cell migration, and cell spread. For cancer gene therapy, one of the most important problems is low gene transfection efficiency.

Methods: In the present study, to take full advantage of adenovirus in gene expression, we prepared mannan-modified recombinant adenovirus using the PTEN gene (Man-Ad5-PTEN) and investigated the effect of Man-Ad5-PTEN combined with docetaxel (Man-Ad5-PTEN-docetaxel) on tumor growth in a murine model of hepatocellular carcinoma.

Results: Man-Ad5-PTEN effectively suppressed tumor growth and induced significant apoptosis of murine H22 hepatoma in vivo. Apoptosis levels in tumor-bearing mice treated with Man-Ad5-PTEN-docetaxel were significantly higher than those in tumor-bearing mice treated with naked Ad5-PTEN, Man-Ad5-PTEN, or docetaxel alone. Treatment with Man-Ad5-PTEN-docetaxel resulted in a significant inhibitory effect in this tumor model. Compared with the controls treated with phosphate-buffered solution, the tumor inhibition rate with naked Ad5-PTEN, docetaxel, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel was 48.69%, 49.98%, 75.88%, and 96.93%, respectively.

Conclusion: These results suggest that combined treatment with Man-Ad5-PTEN and other chemotherapeutic agents may be a potent adjuvant therapeutic approach for the treatment of hepatocellular carcinoma.

Keywords: cancer gene therapy, adenovirus vector, PTEN gene, mannan, docetaxel

Introduction

Viral and nonviral methods of gene delivery have been used in clinical trials, in which viral vectors such as retroviruses, adenoassociated viruses, herpes simplex viruses, and adenoviruses are the most promising vectors for high transfection efficiency. Among these viral vectors, the replication-deficient adenovirus has become one of the most promising gene delivery vectors due to its many advantages, including being easily produced in high titers, an ability to transfect nonproliferating cells readily, no integration into the host genome, and broad tissue or cell tropism. However, the broad host range of the adenovirus is also a disadvantage and can lead to lack of tissue specificity and poor safety. Therefore, it is necessary to improve this therapeutic strategy by improving the cancer-targeting ability of the adenovirus vector. Previous studies have developed strategies using heterologous retargeting complexes, such as chimeric fusion proteins and bispecific antibodies, or genetic capsid modification.
Hepatocellular carcinoma is an aggressive and rapidly fatal malignancy of the liver and is the third leading cause of cancer-related death worldwide. Conventional treatment for hepatocellular carcinoma includes surgery, radiotherapy, and chemotherapy. Although remarkable advances have been made in the clinical study of hepatocellular carcinoma, only a definitive subset of cases is cured, and the overall dismal outcome of patients with hepatocellular carcinoma has not changed, so it is essential that we find a more effective way to treat this disease. Gene therapy represents an exciting biotechnological advance that may revolutionize conventional cancer treatment, and combination treatment using gene therapy and chemotherapy is being paid much attention.

The phosphatase and tensin homolog deleted on chromosome ten (PTEN) gene is a tumor-suppressor gene located on human chromosome 10q23.3, originally identified by two research groups in 1997. Since then, a significant number of PTEN deletions and mutations have been reported in many malignant tumors, including glioblastoma, prostate cancer, melanoma, endometrial carcinoma, lung carcinoma, and hepatocellular carcinoma. PTEN has been reported to play a role in apoptosis, cell cycle arrest, proliferation, cell migration, angiogenesis, and overall survival. Overexpression of PTEN in cancer cells carrying mutant-type or deletion-type PTEN can inhibit cell proliferation and tumorigenicity via induction of cell cycle arrest at the G1 phase and apoptosis. Loss of PTEN expression has been reported to be strongly correlated with aggressive tumor behavior, eg, loss of nuclear PTEN expression was found to be associated with liver metastasis. Restoring PTEN expression in PTEN-deficient tumor cells has been shown to increase radiosensitivity in vivo. Furthermore, previous studies have shown that PTEN is involved in drug resistance in several types of cancer, and cell lines with a high expression of PTEN are sensitive to chemotherapy with 5-fluorouracil and oxaliplatin. Therefore, in this work, we investigated whether the PTEN gene transfected by adenovirus could effectively suppress hepatocellular carcinoma in vivo.

On the other hand, among those targeting strategies mentioned above, conjugating the ligand of the receptor to vectors of interest is an attractive approach. Moreover, it was reported that there is considerably high expression of the mannose receptor in liver nonparenchymal cells and endothelial sinusoidal cells, and that mannose receptors are involved in the binding and subsequent endocytosis of mannose-containing glycoproteins to and into cells. One study showed that plasmid DNA complexed with mannosylated liposomes had high transfection activity due to recognition by mannose receptors both in vitro and in vivo. Another study showed that conjugation of mannann with a recombinant antigen, mucin-1, gives rise to a high cellular immune response, leading to total resistance of experimental tumors in mice.

Putting all this information together, in the present study, to test whether the combination strategy of cancer gene therapy and chemotherapy could enhance antitumor effects, conjugates of mannan-modified Ad5-PTEN (Man-Ad5-PTEN) were prepared and used to treat H22 tumor-bearing mice, together with docetaxel, a well known chemotherapeutic agent. After treatment, tumor volume and weight, along with apoptosis level, were investigated.

**Materials and methods**

**Mice and cell culture**

Male Kunming mice, aged 6–7 weeks, were purchased from the Laboratory Animal Center of Luzhou Medical College, Luzhou, China. The study was approved by the animal ethics committee of Luzhou Medical College, and all procedures with the animals were conducted according to the guidelines of the local animal use and care committees of Luzhou and performed according to the National Animal Welfare Law of China.

Murine H22 hepatoma cells (syngeneic with the Kunming mouse strain) and human embryonic kidney (HEK) 293 cells were sourced from the Chinese Academy of Sciences, Shanghai, China, and maintained in our laboratory. The H22 and HEK 293 cells were, respectively, cultured in RPMI 1640 medium and Dulbecco’s Modified Eagle’s medium (HyClone Corporation, Logan, UH) supplemented with 10% fetal bovine serum (HyClone Corporation), 100 units/mL penicillin, and 100 μg/mL streptomycin, and cultured at 37°C in air containing 5% CO₂.

**Adenoviral vectors**

Replication-deficient human type 5 adenovirus with the expressing reporter gene for β-galactosidase (Ad5-LacZ) was purchased from the Vector Gene Technology Company Limited (Beijing, China), and another adenovirus carrying the PTEN gene (Ad5-PTEN) was a gift from the National Key Laboratory of Biotherapy, Sichuan University, China. Ad5-PTEN was constructed according to a report published elsewhere. Briefly, it was constructed by subcloning full-length PTEN cDNA from a plasmid construct (pCDNA3-PTEN) into a shuttle plasmid containing CMV promoter and SV40 poly (A) elements. The resulted PTEN-containing...
plasmid was cotransfected with pJM17 into HEK 293 cells to obtain the Ad-PTEN virus. The resulting Ad5-PTEN virus was propagated in HEK 293 cells and purified by two rounds of CsCl (Amresco Inc, Solon, OH) density gradient centrifugation. The genome copy number of adenovirus stock was determined by Taqman real-time polymerase chain reaction, and the adenovirus titer was detected using a plaque-forming assay on HEK 293 cells.

Mannan and adenovirus conjugation
Mannan was conjugated to adenovirus in the following manner. Briefly, mannann (Sigma, St Louis, MO), at 25 mg/mL in 0.1 M phosphate buffer (pH 6.0) optimized by periodic acid-Schiff (PAS) stain, was oxidized with sodium periodate (0.01 M, Sigma) for 60 minutes at 4°C. The mixture was desalted by dialysis at 4°C for 5 hours against bicarbonate buffer (pH 9.0) and replaced with fresh buffer every other 30 minutes. The resulting oxidized mannann was mixed with Ad5-PTEN, incubated overnight at room temperature, and used without further purification.

In vivo animal tumor model experiment
H22 tumor cells were obtained for inoculation from in vivo passage. Briefly, the stocked murine hepatoma H22 cells were first subcultured in RPMI 1640 containing 10% fetal bovine serum and collected in blank RPMI 1640 culture medium (1 × 10^6/mL). About 0.2 mL of preformed cell suspension (including 2 × 10^7 cells) was injected into the abdominal cavity of the mouse. After 7 days, the mouse had developed an ascites tumor and was sacrificed by nitrogen inhalation. The tumor burdens from each mouse were weighed and immediately fixed in 4% buffered paraformaldehyde overnight for hematoxylin and eosin (H&E) staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

H&E staining and TUNEL assay
After being treated for 2 weeks, the tumor-bearing mice were sacrificed and the tumor burdens from each mouse were weighed and immediately fixed in 4% buffered paraformaldehyde overnight for hematoxylin and eosin (H&E) staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

All the mice were monitored daily after treatment for tumor size. The tumor measurements were converted to tumor volume (V) by the formula (L × W^2 × 0.52) and expressed as the mean ± standard deviation where L and W are the length and width, respectively. The tumors were measured with a vernier caliper every day until day 19 when the mice treated with phosphate-buffered saline began to die, and then all the mice were sacrificed by nitrogen inhalation. The tumor burden of each mouse was weighed.
Annexin V-FITC was added into each tube. The tubes were then incubated for 30 minutes at room temperature in the dark. The cells were washed with binding buffer and resuspended in 500 µL of binding buffer, with 5 µL propidium iodide added. Finally, the tube was gently vortexed and incubated for another 30 minutes in the dark, and the stained cells were analyzed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) with CellQuest software within one hour.

**Statistical analysis**

The data are shown as the mean ± standard deviation. The significance of the difference between the groups was assessed by the Student’s t-test using SPSS version 16.0 (SPSS Inc, Chicago, IL). In all cases, there was a statistically significant difference ($P < 0.05$, $P < 0.01$, $P < 0.001$).

**Results**

**Characterization assay on Man-Ad5-PTEN by PAS staining**

Man-Ad5-PTEN was prepared under the appropriate oxidizing conditions as described earlier (Figure 1). First, mannan was oxidized by sodium periodate to form oxidized mannan (Ox-Man). The Ox-Man with its aldehyde functional groups was able to bind the amino groups anchored on the surface of the adenovirus by Schiff’s base reaction, which led to formation of Man-Ad5-PTEN. The efficacy of conjugation was confirmed by PAS staining. The PAS stain is a histochemical reaction commonly used to detect glycogen and other polysaccharides in biological specimens. The reaction of periodic acid oxidizes the diol functional groups in glucose and other sugars, creating aldehydes that react with the Schiff

![Figure 1: Synthesis scheme of conjugation strategy for mannan to adenovirus under the appropriate oxidizing conditions.](https://www.dovepress.com/for-personal-use-only)
A medium concentration of free mannan (Figure 2A) was a darker magenta color, whereas the well containing the product resulting from a medium concentration of mannan conjugated with adenovirus showed an immeasurable clear color when stained with PAS (Figure 2E). In contrast, the well containing a high concentration of mannan-modified Ad5 showed a moderate color (Figure 2F). The well containing free Ad5 (Figure 2C) and the well containing a low concentration of mannan-modified Ad5 (Figure 2D) showed no color. The inconspicuous light color observed in the well shown in Figure 2E indicates that the redundant Ox-Man is relatively less likely to react with the PAS reagent. Meanwhile, in our preliminary experiment (data not shown), we used mannan at different concentrations to react with Ad5, and the resulting products were subjected to PAS staining. The absorbance of the mixture was read at 544 nm using a microplate reader (model-550, BioRad, Hercules, CA) after PAS staining. The results indicate that when the concentration of mannan was less than 25 mg/mL, the PAS stain (Figure 2D) was negative and the absorbance in each mixture showed no differences compared with the negative control of PAS reagent alone. Increasing the concentration of mannan to 25, 30, 35, 40, 45, and 50 mg/mL, the absorbance of the mixture also increased and Figure 2F is a representative image. Therefore, we took the corresponding amount as the optimal concentration of mannan conjugated with adenovirus. For visualization, the wells shown in Figure 2A and F were also observed under light microscopy (AL and FL). All the results confirmed the formation of mannan-adenovirus conjugates.

Combination treatment significantly decreased tumor volume

To determine the effects of naked Ad5-PTEN, docetaxel, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel on tumor development in mice, we constructed a mouse tumor model and a single injection of each formulation was administered intratumorally. Among them, one control group was treated with 0.1 mL of phosphate-buffered saline, and the other four groups of mice were treated with Man-Ad5-PTEN, docetaxel, or Man-Ad5-PTEN-docetaxel in a total amount of 0.1 mL.

As shown in Figure 3, there was no therapeutic effect in the control group receiving phosphate-buffered saline, which was consistent with the untreated group (data not shown). Tumor growth in mice treated with Man-Ad5-PTEN-docetaxel was significantly inhibited compared with the other groups of animals treated with phosphate-buffered saline, naked Ad5-PTEN, or docetaxel alone, respectively (P < 0.01). Tumor volume
was suppressed by both Man-Ad5-PTEN and Man-Ad5-PTEN-docetaxel. Compared with the group treated with Man-Ad5-PTEN-docetaxel, the mice treated with Ad5-PTEN or docetaxel appeared to be moderately inhibited in tumor growth ($P < 0.05$). There was no difference between the groups treated with docetaxel or Ad5-PTEN ($P > 0.05$). A clear difference was observed between the Man-Ad5-PTEN-docetaxel and Man-Ad5-PTEN treated groups from day 14 post-injection. When the Ad5-PTEN or docetaxel groups were compared with the combination group, significant differences were also observed ($P < 0.05$).

These results indicate that combination treatment of Man-Ad5-PTEN-docetaxel was the most effective of these formulations, suggesting that Man-Ad5-PTEN can synergize with docetaxel to improve the antitumor effect in H22 tumor-bearing mice after intratumoral administration.

**Combination strategy significantly decreases tumor weight**

To demonstrate the tumor growth inhibition effect more clearly, the H22 tumor-bearing mice were first given the formulation of Ad5-PTEN, docetaxel, PBS, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel. Then, after two weeks, the mice were euthanized by nitrogen inhalation, the tumors were removed and weighed, and the mean tumor weight was calculated. The tumor inhibition rate was calculated using the following formula:

\[
\text{Tumor inhibition rate} = \left(\frac{\text{mean tumor weight of control group} - \text{mean tumor weight of treated group}}{\text{mean tumor weight of control group}}\right) \times 100\%.
\]

The results are shown in Figure 4I and II.

Figure 4 indicates that the average tumor weight of the mice treated with phosphate-buffered saline (Figure 4IA), docetaxel (Figure 4IB), Ad5-PTEN (Figure 4IC), Man-Ad5-PTEN (Figure 4ID), and Man-Ad5-PTEN-docetaxel (Figure 4IE) was $4.8827 \pm 0.3942$ g, $2.4424 \pm 0.2860$ g, $2.5053 \pm 0.2274$ g, $1.1779 \pm 0.2008$ g, and $0.1499 \pm 0.0123$ g, respectively. There is a significant difference between Figure 4IA and IB ($P < 0.05$), Figure 4IA and IC ($P < 0.05$), Figure 4IA and ID ($P < 0.01$), and Figure 4IA and IE ($P < 0.001$). Compared with the control group receiving phosphate-buffered saline, the tumor inhibition rate in Figure 4IB, IIC, IID, and IIE was 49.98%, 48.69%, 75.88%, and 96.93%, respectively. These results demonstrate that combination treatment with Man-Ad5-PTEN and docetaxel significantly decreased the tumor weight in H22-tumor bearing mice.

**Effect of combination treatment on tumor issues analyzed by FACS**

We found that all four treatments could suppress tumor growth to varying degrees compared with the control group receiving phosphate-buffered saline, so we wanted to study the likely mechanism of suppression. To investigate
Man-Ad5-PTEN and docetaxel improve antitumor efficacy

To investigate qualitatively whether the treatments induced cell necrosis and apoptosis in H22 tumor cells, all the groups of tumor-bearing mice were treated intratumorally on day 6. Two weeks later, mice were euthanized by nitrogen inhalation, and the tumors were removed and cut into 5 μm slices. The slices were then prepared for subsequent H&E staining and Tunel assay. The H&E staining results (Figure 6A and B) indicate that tumor cells from the treated mice showed the typical morphology of apoptosis, i.e., nuclear fragmentation, chromatin condensation, and loss of membrane asymmetry. They also showed that the treated groups had increased apoptotic and necrotic cells compared with the control group treated with phosphate-buffered solution. Both the apoptotic and necrotic cell populations in the Ad5-PTEN and docetaxel groups were much smaller than those in the Man-Ad5-PTEN and Man-Ad5-docetaxel groups. Moreover, there were fewer apoptotic and necrotic cells in the Man-Ad5-PTEN group than in the group receiving Man-Ad5-PTEN-docetaxel.

Cellular apoptosis in tumor samples derived from the control and treated mice was also examined using the in situ TUNEL method. The results are shown in Figure 6C, and indicate that the number of apoptotic cells was dramatically increased in tumors treated with Man-Ad5-PTEN and Man-Ad5-PTEN-docetaxel when compared with those in tumors treated with the control phosphate-buffered solution. The number of TUNEL-positive cells was not different between tumors treated with naked Ad5-PTEN and those treated with docetaxel. These results were consistent with those from H&E staining. All the results were consistent with the tumor growth effect in the different groups, suggesting that the apoptotic effect led to tumor growth inhibition.

Discussion

The success of gene therapy in vivo relies on development of a vector that achieves targeted cell-specific, efficient, and prolonged transgene expression after its application. Currently, recombinant adenovirus vectors represent one of the most efficient delivery vehicles for gene therapy in cancer.
Their translocation from the cell surface to the nucleus is extremely rapid and far more efficient than nonviral gene delivery vectors. However, adenoviral vectors show strong antigenicity as a result of their capsid domains, leading to decreased expression of the target gene constructed in the adenoviral vector, along with a need for increasing frequency of administration. Moreover, transfer of adenovirus has limited tissue specificity.

Numerous strategies have been developed to address the abovementioned issues, including second-generation and third-generation adenoviral vectors, fiber-mutated or pentone-mutated recombinant adenoviral vectors, conjugated adenovirus, and some ligands to modify adenoviral tropism and reduce vector-related toxicity. On the other hand, active targeting delivery systems have become a focus in cancer therapeutics, and some targeting ligands, such as aptamer, transferrin, antibody, and folate, are used to modify the vectors of interest for binding to particular receptors overexpressed by tumor cells or tumor vasculature. In this work, in order to block native tropism, extend blood residence time, and enhance tumor targeting, we conjugated adenovirus with mannan under appropriate oxidizing conditions to enable formation of a mannan-modified adenovirus based on the fact that mannose receptors are extensively expressed in nonparenchymal liver tissue. Thus, introduction of mannose residues may lead to enhancement of endocytosis mediated by these receptors. Compared with naked Ad5-PTEN, Man-Ad5-PTEN significantly suppressed growth of H22 tumors, indicating that the mannan and adenovirus conjugation strategy indeed played an important role. The cytotoxicity of Ad5-PTEN or preformed Man-Ad5-PTEN at a variable multiplicity of infection (80, 40 and 20 plaque-forming units/cell) was assessed by MTT viability assay in normal Chang hepatocytes (data not shown). No cytotoxicity was observed in the group treated with naked Ad5-PTEN or in the group treated with Man-Ad5-PTEN, even at the highest multiplicity of infection (80 plaque-forming units/cell), taking the phosphate-buffered solution group as the control. This result suggests that both Ad5-PTEN and Man-Ad5-PTEN may not be cytotoxic to normal cells in vivo. To ensure that chemical modification did not damage the gene expression mediated by Ad5, we investigated transgene expression in vitro using the LacZ reporter gene. The experiment was carried out according to our previous report whereby murine

**Figure 5** Flow cytometry analysis of cell apoptosis in murine hepatoma H22 cells obtained from tumor-bearing mice. The different groups of mice were treated with Ad5-PTEN, docetaxel, phosphate-buffered solution, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel, respectively. The single cell solution was prepared as described in the Materials and methods section and then stained with Annexin V and propidium iodide. The samples were analyzed by FACSCalibur within one hour. The percent of apoptotic cells was compared between the phosphate-buffered solution group (C) and Ad5-PTEN (A), docetaxel (B), Man-Ad5-PTEN (D), and Man-Ad5-PTEN-docetaxel (E).

**Notes:** The significance of the differences was evaluated using the Student’s t-test (*P < 0.05; **P < 0.001). Data are expressed as the mean ± standard deviation (n = 3) in each group.

**Abbreviations:** FITC, fluorescein isothiocyanate; Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten.
Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common cause of cancer mortality. The conventional therapeutic strategy for hepatocellular carcinoma is surgery, chemotherapy, and radiotherapy. However, the cancer recurs and metastasizes in more than one third of patients after conventional therapy. Currently, combined traditional treatment such as chemotherapy and radiation with vaccines has attracted increasing attention. Vaccination can be quite effective in protecting naive mice from tumor challenge and for delaying tumor growth in mice bearing minimal residual disease, but is less effective when given to mice with established tumors. On the other hand, it has been reported that the cancer-suppressing gene, PTEN, plays a critical role in controlling tumorigenesis and regulating cell spread and migration, and adenovirus-mediated PTEN gene therapy is an attractive strategy. However, the ability of single gene therapy alone to suppress tumor growth is limited. To achieve an improved chemotherapeutic effect, a combination of two or more different agents according to their individual mechanisms of action has already been demonstrated to be a popular and effective therapeutic strategy. In a previous study, we reported that combining carmustine chemotherapy with adenovirus-mediated stromal cell-derived factor-1α gene therapy could be successful in the treatment of the murine B16 melanoma.33 Thus, in this study, we developed a mannan receptor-mediated chemoadenovirus-PTEN therapy strategy and combined this with docetaxel, a conventional chemotherapy agent, and PTEN, a tumor suppressor gene.

To investigate the inhibitory effect on tumor growth directly in an empirical manner, we developed a tumor model in vivo and administered Ad5-PTEN, docetaxel, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel by the intratumoral route. To ensure uniform distribution of the formulation in tumor tissue, a single intratumoral injection was carried out in a multipoint manner, whereby pinpoint with fluid does not quit the tumor tissue during one single injection. The results indicate that treatment with Ad5-PTEN and with docetaxel alone could only moderately inhibit tumor growth in H22-bearing mice. However, a combination of Man-Ad5-PTEN and docetaxel dramatically suppressed tumor growth and induced high levels of apoptosis.

The present study is, to our knowledge, the first to combine adenovirus-mediated PTEN gene therapy with docetaxel for cancer treatment. However, the results we obtained are still far from our goal of complete inhibition of tumor growth. Even now, the antitumor mechanism of this combination treatment is still not very clear and requires further investigation. In future studies, we will further optimize the ratio of Ad5-PTEN particle numbers and docetaxel, and also...
investigate the use of combination strategies in hepatocellular carcinoma and other tumors by intravenous administration and elucidate clearly the mechanism of combination therapy. Meanwhile, in the present study, Man-Ad5-PTEN was injected intratumorally and showed obvious tumor growth inhibition. In the next study, we plan to use this approach for in situ liver cancer by intravenous administration. High targeting efficiency mediated by the mannose receptor and strong antitumor activity are expected.

**Conclusion**

Overall, this study demonstrated that Man-Ad5-PTEN significantly enhanced the antitumor effect in a murine model of hepatocellular carcinoma in vivo. Combination treatment of Man-Ad5-PTEN and docetaxel had a better antitumor effect than either strategy used alone. Because of its simplicity, this method may be used to target therapy in other kinds of tumors by intratumoral or intravenous administration. It can be concluded that the combined use of Man-Ad5-PTEN and docetaxel may be a powerful and effective tool for future exploration in tumor therapy.

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**Disclosure**

The authors report no conflicts of interest in this work.

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