β-Mannosylceramide Activates Type I Natural Killer T Cells to Induce Tumor Immunity without Inducing Long-Term Functional Anergy

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Abstract

**Purpose:** Most studies characterizing antitumor properties of invariant natural killer T (iNKT) cells have used the agonist, α-galactosylceramide (α-GalCer). However, α-GalCer induces strong, long-lasting anergy of iNKT cells, which could be a major detriment for clinical therapy. A novel iNKT cell agonist, β-mannosylceramide (β-ManCer), induces strong antitumor immunity through a mechanism distinct from that of α-GalCer. The objective of this study was to determine whether β-ManCer induces anergy of iNKT cells.

**Experimental Design:** Induction of anergy was determined by ex vivo analysis of splenocytes from mice pretreated with iNKT cell agonists as well as in the CT26 lung metastasis in vivo tumor model.

**Results:** β-ManCer activated iNKT cells without inducing long-term anergy. The transience of anergy induction correlated with a shortened duration of PD-1 upregulation on iNKT cells activated with β-ManCer, compared with α-GalCer. Moreover, whereas mice pretreated with α-GalCer were unable to respond to a second glycolipid stimulation to induce tumor protection for up to 2 months, mice pretreated with β-ManCer were protected from tumors by a second stimulation equivalently to vehicle-treated mice.

**Conclusions:** The lack of long-term functional anergy induced by β-ManCer, which allows for a second dose to still give therapeutic benefit, suggests the strong potential for this iNKT cell agonist to succeed in settings where α-GalCer has failed. Clin Cancer Res; 1-8. ©2013 AACR.

Introduction

Natural killer T (NKT) cells are a unique lymphocyte population expressing phenotypic and functional characteristics of both T and natural killer (NK) cells (1, 2). They express true T-cell receptors (TCR) that recognize antigens presented by a nonclassical MHC class Iib molecule CD1d. NKT cells can be subdivided into at least two distinct groups, type I and type II, on the basis of TCR expression (3). Type I or invariant NKT cells (iNKT) cells express an invariant TCR α chain, Vα14-Jα18 in mice (Vα24-Jα18 in humans), and use a limited Vβ repertoire (Vβ2, 7, and 8.2 in mice and Vβ11 in humans). Unlike conventional T cells that recognize peptide antigens, NKT cells mainly recognize glycolipid antigens. The prototypical antigen for iNKT cells is α-galactosylceramide (α-GalCer), which has been shown to be a potent agonist for iNKT cells in both mice and humans (4–6). Upon TCR ligation, iNKT cells rapidly produce large amounts of various cytokines, such as IFN-γ, interleukin 4 (IL-4), IL-13, and TNF-α, and have been shown to induce immunity against tumors and pathogens through the activation of effector cells such as macrophages, NK, and CD8+ T cells (7–10).

iNKT cells have been found to induce potent antitumor immune responses in many different mouse tumor models (10–16). These preclinical studies prompted several human clinical trials with α-GalCer that, to date, have had limited success (reviewed in ref. 17). One potential problem for using α-GalCer to induce clinical responses is the induction of iNKT cell anergy. For conventional T cells, anergy is characterized by impaired proliferation and cytokine production following chronic TCR signaling or signaling in the absence of costimulation (18). In contrast, a single injection of α-GalCer induces iNKT cells to become anergic and unable to produce the same response to restimulation for more than 2 months (19, 20). Multiple injections of α-GalCer do not completely inhibit all cytokine production but instead skew the cytokine response toward Th2 (21), which may not be beneficial in the tumor setting as α-GalCer relies on IFN-γ to induce tumor protection (8, 9, 16).
Translational Relevance

Activation of iNKT cells with α-galactosylceramide was very successful in preclinical mouse models of cancer; however, its success in clinical trials has been very limited. It has been very well documented that, when iNKT cells are activated with α-galactosylceramide, they remain unresponsive to restimulation for months. This functional anergy could be a contributing factor to the failure of α-galactosylceramide clinically, as most therapeutics require multiple dosing to achieve maximum benefit. Here, we report that a different iNKT cell agonist, β-mannosylceramide, which is capable of inducing tumor immunity similarly to α-galactosylceramide but by a different mechanism, does not induce long-term anergy. This suggests that β-mannosylceramide has the potential to work well clinically as it can be given in multiple doses without inducing long-term anergy.

Recently, we described a new antigen for iNKT cells, β-mannosylceramide (β-ManCer), which contains the same ceramide structure as α-GalCer but with a β-linked mannose sugar instead of an α-linked galactose (12). Like α-GalCer, β-ManCer activates iNKT cells to induce an antitumor immune response; however, unlike α-GalCer, which requires IFN-γ for tumor elimination, β-ManCer can protect in the absence of IFN-γ and, instead, requires TNF-α and nitric oxide synthase, suggesting that β-ManCer is the first representative of a new class of agonists for iNKT cells that protect by a different mechanism. Although β-ManCer induced tumor protection comparable with that induced by α-GalCer, other markers of cell activation such as cytokine production and upregulation of activation markers were markedly lower following activation with β-ManCer. Because long-term anergy induction by α-GalCer may be a result of strong activation of iNKT cells, and β-ManCer did not seem to be as strong an inducer of activation of iNKT cells as α-GalCer, we hypothesized that it might not induce the same long-term anergy of iNKT cells as observed following activation with α-GalCer. In assessing β-ManCer for possible clinical development, we investigated whether its use also was limited by induction of anergy.

In this study, we show that β-ManCer does not induce long-term anergy of activated iNKT cells. Instead, iNKT cells activated by β-ManCer are able to respond to restimulation more quickly after the first stimulus and to protect against cancer in vivo even after previous treatment with β-ManCer. The lack of anergy induction is correlated with the level of PD-1 expression on iNKT cells. Because β-ManCer, unlike α-GalCer, activates iNKT cells without inducing a strong persistent anergy, it may have the potential to be repeatedly administered in human cancer patients to induce more effective tumor elimination.

Materials and Methods

Mice

Female BALB/c mice were purchased from Animal Production Colonies, Frederick Cancer Research Facility, NCI. Female mice older than 6 weeks of age were used for all experiments. All experimental protocols were approved by and carried out under the guidelines of the National Cancer Institute's animal care and use committee.

Reagents

α-GalCer (KRN7000) was purchased from Funakoshi (Tokyo, Japan). β-ManCer was synthesized as described previously (12).

Cell lines

The CT26 colon carcinoma cell line was maintained as previously reported (12).

In vitro proliferation and cytokine secretion assay

Unless otherwise indicated, mice were injected with 2 μg (~2.4 nmol) α-GalCer or β-ManCer or vehicle control (PBS containing 0.01% Tween20) intraperitoneally (i.p.). Two months later, mice were sacrificed and splenocytes were harvested and stimulated with 100 nmol/L α-GalCer or 500 nmol/L β-ManCer or vehicle control. After 48 hours of stimulation, supernatants were collected, and the concentrations of IFN-γ, TNF-α, and IL-4 were determined by ELISA. For the proliferation assay, 1 μCi of [3H]-thymidine was added to each well during the final 8 hours of a 72-hour culture, and [3H]-thymidine incorporation was evaluated with a MicroBeta counter (Perkin Elmer).

In vitro proliferation of Vβ subsets of iNKT cells

Splenocytes were harvested from mice 4 weeks after receiving 2 μg (~2.4 nmol) of glycolipid as described earlier. Cells were labeled with 0.1 μmol/L carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 15 minutes at room temperature. Labeled cells (4 × 10^6/well of a 24-well plate) were stimulated for 3.5 days with glycolipid (100 nmol/L α-GalCer or 500 nmol/L β-ManCer) or vehicle control. At the end of the culture, cells were harvested and stained with PBS57-loaded CD1d tetramer (NIH Tetramer Facility), anti-CD3 (BioLegend), and Vβ2, Vβ7, and Vβ8.1/8.2 (BD Biosciences). The fluorescence of stained cells was measured by FACSCalibur (BD Biosciences), and data were analyzed by FlowJo (Tree Star).

iNKT cell and DC activation analysis

Mice were injected with 2.4 nmol (~2 μg) α-GalCer or β-ManCer or vehicle control (PBS containing 0.01% Tween20) i.p. Splenocytes were harvested at the indicated time points and stained with PBS57-loaded CD1d tetramer (NIH Tetramer Facility); anti-CD3, CD40, CD69, and CD86 (BioLegend); PD-1, PD-L1, PD-L2, CD25, CD11b, and CD11c (eBioscience); and CD80 (BD Bioscience). The fluorescence of stained cells was measured by FACSCalibur or LSRII (BD Biosciences), and data were analyzed by FlowJo (Tree Star).
In vivo lung metastasis assay
Mice were injected with 2 μg (2.4 nmol) α-GalCer or β-ManCer or vehicle i.p., and splenocytes were harvested 1 or 2 months after injection and restimulated in vitro with either α-GalCer or β-ManCer. Even in mice pretreated with vehicle, β-ManCer induced less proliferation, IFN-γ, IL-4, and TNF-α production than α-GalCer (Fig. 1A–D), but significantly greater than nonstimulated controls. Consistent with previous reports (23, 24) using the same dose, splenocytes from mice pretreated with α-GalCer were far less able to respond to a second stimulation with either α-GalCer or β-ManCer by any of the indicators measured, and this inability to respond lasted at least 1 to 2 months. By 1 to 2 months after glycolipid administration, splenocytes from mice that received β-ManCer could respond just as well to in vitro glycolipid administration as did vehicle-control mice. These data show that, unlike α-GalCer, which induces anergy for at least 2 months, at the same timepoint, iNKT cells from mice stimulated with β-ManCer can respond to restimulation similarly to naïve iNKT cells.

As β-ManCer has weaker potency to induce cytokine production in vitro (12), we used a 5 times higher concentration of β-ManCer (500 μmol/L) compared with α-GalCer (100 μmol/L) for ex vivo stimulation of spleen cells to be able to obtain measurable cytokine production in the culture supernatant, although we administered the same amount (2 μg) of glycolipid in vivo. To exclude the possibility that the lower potential of β-ManCer to induce anergy is due to a lower potency to activate iNKT cells, we titrated the dose of α-GalCer to administer in vivo. Although 2.4 nmol of α-GalCer induced the strongest anergy, significant reduction of IFN-γ production was observed with the cells from mice treated with 0.48 nmol (0.4 μg) of α-GalCer, which is 5-fold lower than 2.4 nmol, 1 month after the treatment (Supplementary Fig. S1). We also compared the effect of 2.4 and 12 nmol (10 μg) of β-ManCer, as 12 nmol is 5-fold higher than the dose used for α-GalCer, to administer in vivo to determine the effect on the cytokine production of iNKT cells upon in vitro stimulation 6 weeks later (Supplementary Fig. S2). There was no significant difference in the production of either IFN-γ or IL-4 of iNKT cells from mice pretreated with the two different doses. Even 12 nmol of β-ManCer did not induce anergy of α-GalCer–reactive iNKT

Statistical analysis
The data were analyzed using the nonparametric Mann–Whitney test by using GraphPad Prism software (version 5; GraphPad software). The data were considered significant at P < 0.05. All experiments were repeated at least twice to confirm reproducibility of results.

Results
β-ManCer does not induce strong, persistent anergy
Mice were injected with 2.4 nmol (~2 μg) of α-GalCer or β-ManCer or vehicle i.p., and splenocytes were harvested 1 or 2 months after injection and restimulated in vitro with either α-GalCer or β-ManCer. Even in mice pretreated with vehicle, β-ManCer induced less proliferation, IFN-γ, IL-4, and TNF-α production than α-GalCer (Fig. 1A–D), but significantly greater than nonstimulated controls. Consistent with previous reports (23, 24) using the same dose, splenocytes from mice pretreated with α-GalCer were far less able to respond to a second stimulation with either α-GalCer or β-ManCer by any of the indicators measured, and this inability to respond lasted at least 1 to 2 months. By 1 to 2 months after glycolipid administration, splenocytes from mice that received β-ManCer could respond just as well to in vitro glycolipid administration as did vehicle-control mice. These data show that, unlike α-GalCer, which induces anergy for at least 2 months, at the same timepoint, iNKT cells from mice stimulated with β-ManCer can respond to restimulation similarly to naïve iNKT cells.

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cells, whereas 2.4 nmol of α-GalCer did induce anergy. In this particular experiment, the response of the cells from β-ManCer–treated mice was weak, probably because of the shorter interval (6 weeks) between in vivo treatment and in vitro stimulation compared with most other experiments done at 2 months after pretreatment. In fact, when we examined the kinetics of in vivo response over a 2-month period after in vivo glycolipid treatment, the cells from β-ManCer–treated mice weakly responded to β-ManCer at 1, 2, and 4 weeks, whereas thosepretreated with α-GalCer remained anergized even at 2 months (Supplementary Fig. S3; effects most clearly seen in bottom panels where data are normalized because of variable background values). In different kinetic experiments, there was some variability in the duration of anergy at the 1-month time point for β-ManCer, so that, sometimes, anergy persisted 1 month and other times not. However, in every experiment, β-ManCer consistently induced anergy at 1 to 2 weeks, and it was always absent at 2 months, whereas anergy induced by α-GalCer consistently persisted at all time points throughout the 2-month experiment. Thus, β-ManCer fails to induce long-term anergy even at a 5-fold higher dose than the dose required for α-GalCer; therefore, the difference in the ability to induce anergy between α-GalCer and β-ManCer cannot be overcome by using a higher dose of β-ManCer corresponding to the higher concentrations used for ex vivo stimulation.

Because iNKT cells can be subdivided according to their Vβ usage, we wanted to rule out the possibility that the lack of prolonged anergy induction by β-ManCer was due to preferential usage of one Vβ subset to recognize β-ManCer. Pretreatment with β-ManCer had little inhibitory effect on proliferation of iNKT cells in response to β-ManCer restimulation. There was no preferential expansion or retention of one Vβ subset, showing that the lack of anergy induced by β-ManCer stimulation of iNKT cells is not due to selective Vβ usage (Fig. 2).

β-ManCer induces less upregulation of PD-1 and activation markers than α-GalCer

Because the upregulation of programmed death-1 (PD-1) and its ligands has been implicated in the induction and maintenance of anergy induced by stimulation of iNKT cells with α-GalCer, we hypothesized that β-ManCer would not induce sustained upregulation of PD-1 and PD-L1 on iNKT cells or PD-L1 and PD-L2 on dendritic cells (DCs). As previously reported by Parekh and colleagues (23), α-GalCer induced significant upregulation of PD-1 on iNKT cells, which lasted for at least 4 weeks (Fig. 3A and B). PD-L1 was also significantly upregulated on iNKT cells at 6 and 24 hours after glycolipid administration but had returned to baseline by 1 week (Fig. 3B). PD-L2 expression on CD11c+CD11b− DCs followed a pattern similar to that of PD-L1 expression on iNKT cells (Fig. 4A). β-ManCer induced far less upregulation of PD-1, PD-L1, and PD-L2 (Figs. 3 and 4). PD-1 and PD-L1 were significantly upregulated on iNKT cells only 24 hours after glycolipid administration and only to a lesser extent. PD-L1 and PD-L2 expression on DCs was upregulated at 6 and 24 hours, but expression was significantly lower than that induced by α-GalCer. Because PD-1 is also known to be a marker of activated cells (25), we also characterized the kinetics of cell-surface expression of other activation markers on iNKT cells and DCs. CD25 and CD69 were upregulated on iNKT cells 6 hours after α-GalCer stimulation and 24 hours after β-ManCer stimulation (Fig. 3C). CD25 and CD69 expression decreased to control levels by 1 week after glycolipid administration, followed by a subsequent increase in CD69 expression at 2 weeks. CD86 expression on CD11c+CD11b− DCs also increased at 6 and 24 hours and returned to baseline by 1 week for both α-GalCer- and β-ManCer–treated mice (Fig. 4A); however, the intensity of CD86 expression was greater on DCs from mice given α-GalCer than those given β-ManCer. There were no changes in expression of CD40 or CD80 on DCs following glycolipid administration (data not shown). Because CD1d is expressed on many different types of APCs, and we do not know which specific cell type is responsible for presenting β-ManCer to iNKT cells, we also determined the expression of PD-L1, PD-L2, and CD86 on CD11b+ cells. Interestingly, the relative increase of PD-L1 following β-ManCer treatment was greatest on CD11b+ cells compared with the other.
Anergy prevents tumor protection 2 months after α-GalCer but not β-ManCer treatment

We have shown that after activation with β-ManCer, iNKT cells are able to respond to a second stimulation with either β-ManCer or α-GalCer to induce proliferation and cytokine production. We used an in vivo assay to determine if these cells were still able to function in protecting against tumors. Mice were challenged with syngeneic CT26 tumor cells i.v. 2 months after glycolipid administration and 50 pmol of glycolipid was administered i.p. within 1 hour of tumor challenge. Both α-GalCer and β-ManCer were able to protect against tumor formation in mice that were pretreated with vehicle or β-ManCer (Fig. 5). Neither α-GalCer nor β-ManCer could induce significant protection in mice that were pretreated with α-GalCer, suggesting that the iNKT cells which were activated with α-GalCer are functionally anergic and cannot respond to a second stimulation for at least 2 months to induce significant protection against CT26 lung tumors. In marked contrast, β-ManCer activation did not lead to functional anergy and the iNKT cells were capable of responding to a second stimulus at both 1 (data not shown) and 2 months following activation to induce significant tumor protection.

Discussion

α-GalCer has been shown to be very potent at inducing antitumor responses in many mouse models, but clinical trials of α-GalCer in human cancer patients have yet to achieve great success (17). Although anergy of human iNKT cells has not been directly addressed, there were some observations of reduced IFN-γ response in the peripheral blood over repeated doses of α-GalCer (26, 27), and such anergy has been well documented to occur in mice (19, 20, 23, 24, 28); therefore, anergy is a potential hindrance for α-GalCer in the treatment of human cancers. Because anergy cannot be induced in vitro with α-GalCer, we cannot test it in humans short of a clinical trial (data not shown). If iNKT cells are unable to respond to restimulation for months, giving multiple doses of glycolipid may not provide any additional benefit. Even if iNKT cells were able to respond to restimulation, a skewing of the cytokine profile away from IFN-γ, as is observed with α-GalCer, may also be problematic. Thus, especially for the treatment of cancer, an ideal agonist would activate iNKT cells without inducing anergy. Weak agonists that do not induce anergy in the past have not been protective either. Here, we report that iNKT cells stimulated with β-ManCer, which is protective, do not remain anergic for months, and are able to respond to restimulation similarly to naïve iNKT cells within 1 to 2 months after the first stimulation, and do not exhibit a strong skewing toward Th2. In addition, their protective efficacy is not dependent on IFN-γ. Most importantly, β-ManCer–activated iNKT cells are able to respond to a second stimulus to induce tumor protection whereas α-GalCer activated iNKT cells cannot. Because most clinical protocols require multiple doses of therapeutics, it would be beneficial to use a compound such as β-ManCer to
activate iNKT cells for which multiple doses could be administered.

In all the methods we used to quantitate anergy, iNKT cells from mice treated with β-ManCer never exhibited as strong or durable an anergic phenotype as those from mice given α-GalCer. Due to the unavailability of reagents, it would be very difficult for us to definitively prove that β-ManCer activates the entire subset of iNKT cells that α-GalCer does. The loss of antitumor activity of β-ManCer in Jα18−/C0−/C0− mice suggests that β-ManCer–reactive NKT cells use the invariant TCR α chain expressed by α-GalCer–reactive iNKT cells (12). In livers, the β-ManCer–loaded CD1d-reactive iNKT cell population is much smaller than that of α-GalCer–loaded CD1d-reactive iNKT cells. These data may suggest that β-ManCer–reactive iNKT cells might be a small subset of α-GalCer–reactive iNKT cells. One can argue that this can be an explanation for the observation that there is no apparent loss of reactivity against α-GalCer in vitro in the splenocytes of β-ManCer–treated mice. However, a second dose of β-ManCer still elicits an effect, not only in vitro but also, importantly, in vivo (see especially the completely undiminished efficacy against cancer of β-ManCer in β-ManCer–pretreated animals in Fig. 5 compared with vehicle-pretreated controls). Thus, even if the repertoire of β-ManCer–reactive iNKT cells is limited, those cells are not becoming anergic after the first dose of β-ManCer and, therefore, the hypothetical possibility that not all iNKT cells recognize β-ManCer cannot be an explanation for the lack of anergy induction by β-ManCer. Furthermore, the lack of functional anergy in cancer treatment in vivo 2 months after β-ManCer pretreatment compared with α-GalCer pretreatment implies that whatever portion of iNKT cells that react with β-ManCer remain able to respond to β-ManCer to be clinically efficacious in cancer therapy even after pre-exposure to β-ManCer, in contrast to those pretreated with α-GalCer.
Although proliferation and cytokine secretion was always less in response to β-ManCer than to α-GalCer, their induction of antitumor immune responses is similar (12), suggesting that such a strong cytokine response by iNKT cells is not required for inducing tumor protection. Thus, while we have shown that β-ManCer induces weak activation of iNKT cells, this may be irrelevant in the context of tumor protection, where β-ManCer is capable of inducing protection without strong activation. It is possible that the less intense signal which β-ManCer provides to iNKT cells prevents the upregulation of molecules that may cause anergy. This quantitative difference, together with the qualitative difference of the β-ManCer effects on iNKT cells, may be the reason for the lack of long-term anergy induction.

Here, the lack of anergy induced by β-ManCer inversely correlated with PD-1 upregulation, which was also much more transient for β-ManCer than for α-GalCer. The immunosuppressive molecule PD-1 and its ligands, PD-L1 and PD-L2, have been implicated in the induction and maintenance of anergy in α-GalCer–activated iNKT cells as upregulation of PD-1 correlates with anergy of iNKT cells (23, 24); however, the exact role of PD-1 and its ligands in α-GalCer–induced anergy remains unclear. A recent report clearly showed that PD-1 is not the sole contributor to anergy in response to α-GalCer stimulation, as anergy still occurred in PD-1 knockout mice (28). In the studies of Parekh and colleagues and Chang and colleagues, blockade of PD-1 and its ligands can overcome anergy induced with α-GalCer. As discussed earlier, previous studies have shown that blockade of PD-1 and its ligands can overcome anergy induced with α-GalCer (23, 24). We propose that, for the purpose of overcoming iNKT cell anergy, using β-ManCer, or a combination of β-ManCer and α-GalCer, is better than a strategy involving PD-1 blockade, as PD-1 blockade will affect far more cell types and pathways than using these glycolipids which target only iNKT cells.

The lack of anergy induction and lower level of PD-1 induction on β-ManCer–activated iNKT cells that we report here might be interpreted as the result of activation of a small fraction of iNKT cells by β-ManCer, based on our previous observation that only a small fraction of iNKT cells can be stained with β-ManCer–loaded CD1d-tetramer (12). However, we believe that this is not the case for the following reasons: first, at the time PD-1 expression was detected on iNKT cells from β-ManCer–treated mice, similar to the cells from α-GalCer–treated mice, the entire iNKT cell population shifted to higher intensity of PD-1 staining, although the magnitude of the shift was smaller in those pretreated with β-ManCer. If only a fraction of iNKT cells were activated, the majority of cells should not shift. Second, if only a fraction of iNKT cells were activated and were anergized and the rest of the iNKT cells could not respond to β-ManCer, there should be no protection against tumors in β-ManCer–treated mice that had been pretreated with β-ManCer. Third, our hypothesis is supported by the observation that β-ManCer and α-GalCer induce iNKT cell proliferation measured by CFSE dilution in a similar proportion of the cells in both mouse and human studies (12). Fourth, in the kinetic experiment shown in Supplementary Fig. S3, at 2 weeks after pretreatment, β-ManCer induces transient anergy of iNKT cells responding to α-GalCer. If only a small subset recognized and were anergized by β-ManCer, no anergy even at early time points should have been seen when stimulating with α-GalCer.

We have previously shown that β-ManCer and α-GalCer induce antitumor responses through different mechanisms and synergize when administered together (12), which suggests great potential for the clinical use of β-ManCer. Moreover, β-ManCer is not a target of natural anti-α-Gal antibodies present in humans but not mice, which may limit efficacy of α-GalCer in humans, and it stimulates human iNKT cells in vitro equally well (12). For these reasons, we investigated the potential of β-ManCer for clinical development. We now show that β-ManCer does not induce a strong skewing of the cytokine profile or long-term anergy of the entire population of iNKT cells, indicating that patients could be treated with multiple doses. These properties make β-ManCer an even more attractive candidate for use in the clinic and suggest that it has the potential to work in cases where α-GalCer does not in treating human cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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