The accumulation of un-repairable DNA damage in laminopathy progeria fibroblasts is caused by ROS generation and is prevented by treatment with N-acetyl cysteine

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Fibroblasts from patients with the severe laminopathy diseases, restrictive dermopathy (RD) and Hutchinson Gilford progeria syndrome (HGPS), are characterized by poor growth in culture, the presence of abnormally shaped nuclei and the accumulation of DNA double-strand breaks (DSB). Here we show that the accumulation of DSB and poor growth of the fibroblasts but not the presence of abnormally shaped nuclei are caused by elevated levels of reactive oxygen species (ROS) and greater sensitivity to oxidative stress. Basal levels of ROS and sensitivity to H₂O₂ were compared in fibroblasts from normal, RD and HGPS individuals using fluorescence activated cell sorting-based assays. Basal levels of ROS and stimulated levels of ROS were both 5-fold higher in the progeria fibroblasts. Elevated levels of ROS were correlated with lower proliferation indices but not with the presence of abnormally shaped nuclei. DSB induced by etoposide were repaired efficiently in normal, RD and HGPS fibroblasts. In contrast, DSB induced by ROS were repaired efficiently in normal fibroblasts, but in RD and HGPS fibroblasts many ROS-induced DSB were un-repairable. The accumulation of ROS-induced DSB appeared to cause the poor growth of RD and HGPS fibroblasts, since culture in the presence of the ROS scavenger N-acetyl cysteine (NAC) reduced the basal levels of DSB, eliminated un-repairable ROS-induced DSB and greatly improved population-doubling times. Our findings suggest that un-repaired ROS-induced DSB contribute significantly to the RD and HGPS phenotypes and that inclusion of NAC in a combinatorial therapy might prove beneficial to HGPS patients.

INTRODUCTION

Laminopathies are a group of inherited degenerative disorders that are caused by mutations in the gene LMNA. This gene encodes the type V intermediate filament proteins lamins A and C, which are produced by alternative splicing. Lamins A and C are essential components of the nuclear lamina in differentiated cells and tissues that have roles in cellular integrity, transcription and cell signalling (1). The most severe laminopathies are progeroid syndromes, which include Hutchinson Gilford progeria syndrome ([HGPS;] 2,3), A-typical Werners syndrome (4), restrictive dermopathy ([RD;] 5) and mandibular acral dysplasia ([MAD;] 6). In the cases of HGPS, RD and MAD, the diseases can also arise as a result of mutations in the gene encoding the ZmpSte24 metalloproteinase (7). Lamina A is first synthesized as a precursor molecule-termed prelamin A, which then undergoes a sequence of post-translational processing steps. Initially, this involves modification of a C-terminal motif CaaX by farnesylation of the cysteine residue, proteolytic cleavage of the three C-terminal amino acids and carboxy methylation of the cysteine residue to give rise to FC-prelamin A. Once localized to the nuclear lamina, FC-prelamin A undergoes a further proteolytic cleavage step to remove the 15 C-terminal peptides (including the farnesylated cysteine residue) to produce mature lamin A (8). The final cleavage step is performed by the ZmpSte24 protease.

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protease (9). A majority of the mutations that give rise to HGPs are caused by silent mutations that activate a cryptic splice site giving rise to a truncated form of lamin A, termed progerin, which can be farnesylated and methylated but which cannot undergo the final cleavage step. Homozygous mutations in ZmpSte 24 leading to loss of its activity cause the accumulation of FC-prelamin A in cells of affected individuals, giving rise to the notion of FC-prelamin A toxicity (10–12). This in turn has led to the use of farnesyl transferase inhibitors (FTIs) as a potential treatment for HGPs (13,14).

It has recently been shown that cells and tissues from mouse models of HGPs, as well as fibroblasts from HGPs, RD and MAD patients accumulate DNA double-strand breaks (DSB) and are sensitized to ionizing radiation (15–18). Initially, it was thought that the presence of progerin or FC-prelamin A in some way impairs the rate of DSB repair (15). However, other studies have revealed that treatment of HGPs and RD fibroblasts with FTIs, while reversing some phenotypic effects of the diseases, does not reduce the load of DSB. These studies suggest that accumulation of DNA damage is itself an important contributor to the diseases and questions the use of FTIs alone as a therapeutic tool (19). This finding is consistent with studies that demonstrate that treatment of progeroid fibroblasts with FTIs activates an alternative geranyl geranylation prelamin A processing pathway and that a combination of statins and FTIs are required for effective geranyl geranylation prelamin A processing pathway and that progeroid fibroblasts with FTIs activates an alternative is consistent with studies that demonstrate that treatment of the use of FTIs alone as a therapeutic tool (19). This finding itself an important contributor to the diseases and questions the use of FTIs alone as a therapeutic tool.

More recently, the Zou group has shown that DNA damage repair foci in progeria fibroblasts are unable to recruit some DNA repair factors including Rad50 and Rad51 but do recruit the nucleotide excision repair protein xeroderma pigmentosa A (21), suggesting that the accumulation of DSB occur because DNA damage repair foci lack function. Remarkably, this study also revealed that DSB induced by genotoxic agents did form functional repair foci and this damage could be repaired, implying a difference between the DSB caused by these agents and those DSB caused by the progeroid laminopathy environment (21).

Recently, it has been shown that progeria fibroblasts maintain elevated levels of reactive oxygen species (ROS) (22,23). Since ROS can act as an endogenous mutagen that causes DSB and is thought to be involved in the accumulation of unrepairable DSB in aged cells (24,25), we speculated that these high levels of ROS were responsible for the DSB, which accumulate in progeroid cells. To investigate this possibility, we compared the repair of DSB induced by either ROS or the genotoxic agent etoposide in dermal fibroblasts from normal young and old individuals and from individuals with RD or HGPs. By fitting a mechanistic model of DSB repair to our data, we show that the rates of DSB repair appear equivalent in all sources of fibroblasts. However, whereas DSB induced by etoposide could be repaired in progeroid fibroblasts, many DSB induced by ROS were un-repairable. Treatment of progeroid fibroblasts with the ROS scavenger N-acetyl cysteine (NAC) reduced the levels of un-repairable DSB and improved their growth rates in culture. Our findings suggest that the ROS-generating environment of progeroid fibroblasts is the primary cause of the accumulation of un-repairable DSB and that the use of ROS scavengers in conjunction with FTIs might improve quality of life for progeria patients.

RESULTS

In order to confirm that RD and HGPs fibroblasts maintain a ROS-generating environment, we measured levels of ROS using the oxy-activatable dye dichlorofluorescein-diacetate (DCF-DA). Basal levels of ROS were more than three times higher in RD and HGPs fibroblasts compared with fibroblasts from normal young (GM) and old individuals (AG). Following stimulation with H2O2, the levels of ROS increased by ~5–10-fold in GM and AG fibroblasts. Following H2O2 stimulation, ROS levels were elevated to even higher levels in both RD and HGPs fibroblasts, which were 4–7-fold greater than normal fibroblasts. Pre-treatment of all cells with NAC prevented ROS stimulation in the presence of H2O2. In addition, 24 h after H2O2 stimulation, the levels of ROS had subsided to basal levels in all cell strains (Fig. 1A).

In a previous study, we have shown that fibroblasts undergo an adaptive response to ROS stimulation, depending upon the level of stress and the lamin mutation harbored by the cell, involving either temporary or permanent cell cycle arrest. Therefore, to determine the cellular responses to ROS stimulation, we first investigated the fraction of proliferating cells before and after H2O2 treatment. The initial rates of cell proliferation did not differ significantly in GM and AG fibroblasts. As expected, the fraction of proliferating cells was almost 50% lower in RD and HGPs fibroblasts compared with GM and AG. Cell proliferation was depressed in all cell lines following H2O2 stimulation, but in the case of RD and HGPs the fraction of proliferating cells was reduced significantly to <10%. These effects were prevented by pre-treatment of cells with NAC. When the fibroblasts were reseeded following ROS stimulation, the proliferation rates recovered in HGPs fibroblasts but not in RD fibroblasts (Fig. 1B). Next we investigated the influence of ROS stimulation on nuclear shape. As expected, in untreated cells the fraction of dysmorphic nuclei was >4-fold higher in RD and HGPs fibroblasts compared with both GM and AG fibroblasts. ROS stimulation had no effect on the fraction of dysmorphic nuclei in any of the fibroblast strains. Neither did NAC treatment in the absence of H2O2 stimulation reduce the fraction of dysmorphic nuclei in any of the fibroblast strains (Fig. 1C). Taken together, these findings suggest that while ROS stimulation does affect cell proliferation, the accumulation of dysmorphic nuclei is independent of the ROS-generating environment of RD and HGPs fibroblasts.

ROS is a known internal mutagen that can generate DNA damage (25). We therefore wondered whether the ROS-generating environment of RD and HGPs fibroblasts might explain the accumulation of DSB reported previously (19). We therefore investigated the accumulation and repair of DSB in response to the genotoxic agent etoposide or ROS stimulation. To do this, we stained fibroblast cultures with antibodies against the phosphorylated form of γH2A.X that detects DSB (26). Cells were fixed and stained at various time points and the numbers of DSB were counted in representative cells. Cells were grouped according to the number of DSB that they contained (Supplementary Material). Since these groupings were to some extent arbitrary, particularly the groups containing the largest number...
of γH2A.X foci and because the time course did not permit us to follow individual cells and DNA damage foci in real time, we developed a mechanistic model of DNA repair and fitted it to our data taking into account all of the associated uncertainties (27,28). Following treatment with etoposide, the initial levels of DSB were higher in RD and HGPS fibroblasts compared with GM and AG fibroblasts; however, all four fibroblast strains were able to repair the induced damage, although the presence of NAC appeared to decrease the rates of DNA repair in all cases (Fig. 2A). DSB also accumulated in all cell strains following ROS stimulation. This damage was efficiently repaired in GM and AG fibroblasts in the presence and absence of NAC (Fig. 2B). In contrast, in RD and HGPS fibroblasts less than half of the DSB were repaired at a rate that appeared consistent with the rate of repair of GM and AG fibroblasts. The remaining DSB were apparently unrepaired during the 24 h of the study. DSB also accumulated in fibroblasts that had been pre-treated with NAC, presumably as a direct effect of the H2O2 (29). NAC pre-treatment of RD and HGPS fibroblasts did, however, permit repair of nearly all of the induced DSB (Fig. 2B). Since ROS-induced DSB did appear to accumulate in RD and HGPS fibroblasts in the absence of NAC, we used the model to infer whether rates of DSB repair slowed or whether un-repairable DNA damage accumulated. Our data did reveal variation in the rates of DSB repair between treatments and, in particular, a consistent reduction in the rate of DSB repair was observed in the presence of NAC. However, 95% confidence bounds on our repair rate estimates and a model selection analysis (Supplementary Material, Table S1) indicate that DSB repair rates did not vary substantially between any of the fibroblasts strains (Fig. 3A). In contrast, a model selection analysis (Supplementary Material, Table S1) and 95% confidence bounds on the number of un-repairable DSB both indicated that ROS stimulation led to the generation of substantially more un-repairable DSB in RD and HGPS fibroblasts. Importantly, the model showed that the presence of NAC almost eliminated this accumulation of un-repairable DSB (Fig. 3B).

Since the data did reveal that NAC pre-treatment permitted DSB repair in the progeria fibroblast strains, we wondered whether prolonged treatment with NAC would reduce the basal levels of DSB and improve growth rates in these cells. Initially, we determined the basal levels of DSB in untreated fibroblasts. Consistent with previous studies (21), we found that the DSB load was significantly higher in untreated RD and HGPS compared with normal GM and AG fibroblasts. The 95% confidence intervals were more variable for HGPS fibroblasts than for RD fibroblasts, reflecting the fact that fewer RD fibroblasts (55%) had no DSB compared HGPS fibroblasts (69%), but there were more HGPS fibroblasts with very large numbers of DSB (Fig. 4A and Table 1). Following, prolonged growth in the presence of NAC, the fraction of RD and HGPS fibroblasts both increased significantly to 72 and 78%, respectively (Fig. 4B). In particular, the HGPS fibroblasts grew very slowly in the absence of NAC, whereas after 2 days growth in the presence of NAC cell proliferation rates increased dramatically (Fig. 4C and D). Taken together, our data suggest that the ROS-generating environment in RD and HGPS fibroblasts leads to the accumulation of DSB, which in turn contributes to the poor rates of growth in these cells. Moreover, treatment of these fibroblasts with NAC reverses both effects.

Figure 1. ROS responses in fibroblasts strains from normal individuals and progeria patients. (A) Dermal fibroblasts from normal young (GM) and old (AG) individuals and from patients with RD and HGPS were treated with H2O (basal) or 150 mM H2O2 in the presence or absence of NAC. ROS generation was determined by DCF fluorescence 30 min after treatment or 24 h after H2O2 treatment to measure recovery after stimulation. Values are based on duplicate FACS measurements in triplicate experiments. (B) The proliferation index of each cell strain was estimated by ki67 expression and expressed as a value relative to the proliferation index in H2O-treated GM fibroblasts. Mean values ± standard error, are based on counting 200 cells on each of two coverslips in triplicate experiments. (C) The effects of ROS on nuclear shape were determined by staining cells with anti-lamin A antibodies in the presence or absence of H2O2 stimulation or in untreated cells grown in the presence of NAC. Mean values ± standard error, are based on 200 nuclei scored on each of two coverslips in triplicate experiments.
DISCUSSION

In this study, we have shown that the ROS-generating environment in RD and HGPS fibroblasts leads to the accumulation of un-repairable DSB. Moreover, the accumulation of ROS-induced DSB appears to be responsible for the poor growth rates of these fibroblasts in culture since treatment of the fibroblasts with the ROS scavenger NAC rescues their ability to repair DSB, reduces the fraction of cells carrying un-repaired DSB and improves their population-doubling time. Our data suggest that the accumulated DSB previously noted to be caused by the laminopathy environment of progeria fibroblasts (19,21) results from the higher basal levels of ROS in those fibroblasts and their greater sensitivity to ROS-inducing agents. Intriguingly, our data also suggest that while lamin A is involved in ROS-induced DNA damage repair, defective lamin A does not appear to influence the repair of DSB induced by genotoxic agents.

Recent studies into the causes of laminopathy progerias have focused on the inability to correctly process lamin A and the consequent accumulation of farnesylated forms of lamin A (30). This in turn has led to the use of FTIs and statins in clinical trials as putative therapies for affected children (31). More recently, a number of studies have questioned whether the partial processing of lamin A is the only cause of progeria and whether the beneficial effects of FTIs and statins is a direct consequence of the depletion of farnesylated forms of lamin A (32). In particular, transgenic knock-in mice expressing a non-farnesylated form of progerin do develop progeria-like phenotypes (33), while administration of FTIs to Caenorhabditis elegans reverses age-dependent nuclear abnormalities without affecting lifespan (34). More recently, the efficacy of long-term administration of FTIs to children has also been questioned since FTIs generate centrosome separation defects in cultured cells (35).

Our data suggest that the ROS-generating environment might be a more significant factor than nuclear shape abnormalities in the development of premature ageing in RD and HGPS patients. Depletion of ROS reduces the DSB load and improves growth rates, without affecting the accumulation of dysmorphic nuclei, in fibroblast models of HGPS. Our data are entirely consistent with other recent studies that have revealed the accumulation of oxidized proteins in fibroblasts from HGPS patients (36) and the lack of correlation of nuclear shape abnormalities with lifespan in C. elegans (34).

Hyperlipidaemia is associated with a number of laminopathy syndromes (37) and the administration of statins in the management of HGPS patients is recommended to control abnormal circulating lipid profiles (38). Recent studies have shown that in chronic diseases caused by a combination of hyperlipidaemia and high levels of ROS, administration of statins and NAC is a highly effective and synergistic combinatorial therapy (39). We propose that inclusion of NAC in combinatorial therapies could prove beneficial to patients with laminopathy progerias.
MATERIALS AND METHODS

Cell culture

Human dermal fibroblasts were cultured in Dulbecco’s modified Eagles medium (DMEM) (Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin at 37°C. Cell stocks were created from cultures obtained either from the Human Genetic Repository (normal individuals) or from the Corielle Cell Repository (RD and HGPS individuals). GM03348 is a fibroblast strain from a healthy 8-year-old male. AG13129 is a fibroblast strain from a healthy 89-year-old man. RD30178 is a fibroblast strain from a RD patient. AG11513 is a fibroblast strain from a HGPS patient. Cells were used between passages 9 and 13. To generate growth curves, cells were seeded at an initial density of $5 \times 10^5$ cells in 35 mM culture dishes. The culture medium was supplemented with either 20 ml/ml of a 10 mM stock solution of NAC or an equivalent volume of dH2O. Fresh culture medium containing NAC or dH2O was added each day. Cells were harvested at daily intervals and counted using a Neuberg Hemocytometer.

Cell treatments with hydrogen peroxide, etoposide and NAC

Fibroblasts were cultured on glass coverslips in six-well plates (four coverslips per well), and allowed to reach 80% confluence. To induce DNA damage two procedures were used: growth medium was removed from cells, and 1 ml phenol-free, low-glucose DMEM was added to each well. Untreated controls did not receive any further treatment. To treated cells, 4 µl of 20 µM etoposide (Sigma) was added per ml of medium, and cells were incubated in the dark for 20 min at 37°C, 5% CO2. After incubation, the media containing etoposide were removed and replaced with normal growth media. Alternatively, growth media were removed from cells, and 1 ml phenol-free, low-glucose DMEM media were added to each well. 37.5 µl of a 4 mM solution of H2O2 was added per 1 ml growth media to give a final concentration of 150 mM. Cells were incubated with the hydrogen peroxide in the dark for 20 min at 37°C. After incubation, the media containing H2O2 were removed and replaced with normal growth media. For pre-treatment with NAC 2 µl of a 10 mM stock solution was added per 1 ml of growth medium and cells were incubated for 1 h at 37°C prior to treatment with either H2O2 or etoposide. Coverslips were removed for fixation and immunofluorescence at various time intervals after treatment.

ROS measurement

Fibroblasts were seeded in a six-well plate, at a density of 50 000 cells per well. The cells were grown in DMEM supplemented growth media and allowed to reach $\sim 70–80\%$ confluence. The growth medium was then removed and replaced with 1 ml of phenol-free, low-glucose DMEM. A 1 mM stock solution of the ROS activated fluorescent dye DCF-DA (Cell Signalling) was prepared in DMSO. Five microlitres of this solution was added per 1 ml of growth medium and cells were incubated for 1 h at 37°C prior to treatment with either H2O2 or etoposide. Cells were harvested at daily intervals and counted using a Neuberg Hemocytometer.

Figure 3. Estimation of the rates of DSB repair and the accumulation of unrepaired DSB. The accumulation of un-repaired DSB (B) and the rates of DSB repair (A) in the presence (closed circles) or absence (open circles) of NAC were estimated from the data shown in Figure 2 using a model of DNA repair and maximum likelihood. Each panel shows the maximum-likelihood parameter estimates and their associated 95% confidence intervals calculated using the profile-likelihood approach.
growth medium and the cells were analysed 24 h later. For analysis, cells were washed twice with phosphate buffered saline (PBS), carefully scraped and suspended in 500 µl PBS in a fluorescence activated cell sorting (FACS) vile. All results were based on triplicate samples obtained in two separate experiments. Cells not treated with dichlorfluorescein-diaceacetate were used to determine auto-fluorescence and deducted from all experimental data. Cells were analysed based on DCF-DA fluorescence using FACScaliber using CellQuestPro software and results were analysed in FlowJo.

Table 1. The load of DSB in different fibroblasts strains

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Mean number of DSB</th>
<th>95% Confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM</td>
<td>0.20</td>
<td>0.14, 0.30</td>
</tr>
<tr>
<td>AG</td>
<td>0.55</td>
<td>0.38, 0.84</td>
</tr>
<tr>
<td>RD</td>
<td>1.73</td>
<td>1.38, 2.20</td>
</tr>
<tr>
<td>HGPS</td>
<td>2.74</td>
<td>1.83, 4.43</td>
</tr>
</tbody>
</table>

The mean number of DSB in GM, AG, RD and HGPS fibroblasts grown under normal culture conditions were calculated by staining cells with anti-γH2AX and counting the number of DNA damage foci in 200 cells on duplicate coverslips and in triplicate experiments. The table shows the estimated mean number of DSB in each cell strain and their associated 95% confidence intervals.

Figure 4. Effects of NAC on the accumulation of DSB and cell proliferation rates in RD and HGPS fibroblasts. (A) The number of DSB in untreated dermal fibroblasts from normal young (GM) and old (AG) individuals and from patients with RD and HGPS were estimated by staining cultures with anti-γH2AX antibodies and counting the number of DNA damage foci in 200 cells on duplicate slides and from triplicate experiments. Cells were grouped according to the number of DNA damage foci they contained (0, 1–2, 3–5, 6–10, 11–15, 15+). The bars represent the mean fraction of cells ± standard error for each group and for each cell strain. (B) Dermal fibroblasts from RD and HGPS patients were cultured in the presence or absence of NAC and stained with anti-γH2AX antibodies. Two hundred cells were scored on duplicate slides and in triplicate experiments to determine the fraction of cells containing no DNA damage foci. Bars show the mean ± standard error for each treatment and each cell strain. Significance was determined by a paired t-test (*P < 0.05, **P < 0.01). (C) RD and (D) HGPS fibroblasts were cultured in the presence or absence of NAC. Cells were harvested each day for 4 days and counted. Mean cell numbers on each day were calculated from triplicate experiments. Significance was determined by a paired t-test (*P < 0.05, **P < 0.01).

Immunofluorescence

Dermal fibroblasts were seeded at a density of 50 000 cells per well of a six-well plate (Sigma), each containing four glass coverslips and allowed to reach 70% confluence. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The coverslips were then washed three times in fresh PBS for 5 min each. Cells were then permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min at 4°C and again washed with PBS. Nonspecific antibody-binding sites were blocked by incubation with 1% FBS in PBS for 45 min at room temperature. Coverslips were then incubated with primary antibodies in new born calf serum for 1 h at room temperature in the dark in moist staining chambers.
After incubation with primary antibodies, coverslips were washed five times in PBS and then incubated with secondary antibodies for 1 h at room temperature in the dark. Coverslips were again washed five times in four PBS and last five times in deionized water. Coverslips were then mounted onto glass slides in 30% glycerol containing Mowiol (Sigma), 2 mg/ml 4,6-diamidino-2-phenylindole and 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma). Images of stained cells were taken using a Bio-Rad Radiance 2000 confocal laser scanner attached to a Zeiss Axioskop. Images were viewed and saved using AxioVision, before importing to ImageJ and Adobe Photoshop 7.0. Primary antibodies used in this study were mouse anti-phospho γH2ÂX (Millipore), rabbit anti-Ki67 (DAKO) and rabbit anti-lamin A (Sigma). Secondary antibodies used in this study were FITC-donkey anti-mouse IgG and TRITC donkey anti-rabbit IgG. All antibodies were used at the manufacturers recommended dilution.

**Model fitting and data analysis**

For each cell line and treatment, the dynamics of DNA repair was modelled using

\[
N(t) = n exp[-r(t - t_1)] + m
\]

where \(N(t)\) is the number of foci at time \(t\), and \(n\) and \(m\) are the expected number of repairable and non-repairable foci induced by the treatment at time \(t_1\), respectively. Here, we have assumed that no additional DSB were induced after time \(t_1 = 0.5\) h, which is the time that DSB data were first collected, and repairable foci were subsequently repaired at constant rate \(r\). To correctly account for variation among cells caused by unmeasured sources, for each cell line and treatment, we assumed that the number of DSB observed among cells varied about the modelled mean [Eq. (1)] according to a negative-binomial distribution. The model parameters \((m, n, r)\) were estimated using maximum likelihood and uncertainty in these parameters was estimated using the profile-likelihood method (40). Model selection incorporating Akaike’s Information Criterion (27,28) was then used to detect evidence that the NAC treatment affected each of the three model parameters; namely, the number of non-repairable and repairable foci induced, and the rate at which repairable foci were repaired (see Supplementary material for full details).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at *HMG* online.

**Conflict of Interest statement.** None declared.

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