Polyamines, such as putrescine, spermidine, and spermine, are polycationic amines that interact with negatively charged molecules (e.g., DNA, RNA, and proteins) to play important roles in a range of biological processes such as cell growth. Present at sub-millimolar concentrations, their expression levels within mammalian cells are known to be tightly regulated by various biosynthetic, degradation, and transport processes. Recent studies have suggested that polyamines (along with other biomolecules such as threonine and highly unsaturated lipids) are implicated in the amine oxidase-based production of acrolein, a highly reactive unsaturated aldehyde, under oxidative stress conditions.

Acrolein, which can also be produced during the burning of organic materials or smoking, has been shown in literature to react with thiol, hydroxyl, or amino functional groups of DNA, proteins, or phosphatidyl ethanolamines to accelerate oxidative stress processes associated with various disease states (e.g., cancer, stroke, arteriosclerosis, or Alzheimer’s disease (AD)). As a consequence, the detection of 3-formyl-3,4-dehydropiperidine (FDP), formed from two molecules of acrolein with the ε-amino group of lysine, is currently employed as an oxidative stress marker. In a similar process, polyamines have also been shown to react with acrolein to produce the corresponding FDP derivatives.

With an increase of cellular polyamine or cytotoxic acrolein levels, research has shown a correlation with the progression of certain diseases, such as cancer or stroke. In the brains of AD patients, observed levels of acrolein or spermine (SPM) are increased whereas spermidine (SPD) or putrescine levels are decreased. Recent reports also indicate that polyamines can promote amyloid-β peptide 1–40 (Aβ40) fibrillation, which is implicated in the acceleration of the AD process.

Recently, we discovered that spermine and spermidine smoothly react with acrolein to produce 1,5-diazacyclooctanes (cyclic spermine and spermidine, cSPM and cSPD) through a formal [4+4] cycloaddition of the intermediary unsaturated imines (Scheme 1). We demonstrated that these compounds are produced in much higher amounts and efficiency than the oxidative stress marker, FDP, which thus far has only been detected under standard analytical conditions. This likely suggests that acrolein reacts with polyamines to exclusively produce the eight-membered heterocycles as initial acrolein-modified intermediate. Given these results, there is considerable potential for these compounds to be implicated in biological processes that were previously unexplored or overlooked. This can be supported by our recent demonstration that diaza heterocycles produced from polyamines (e.g., cSPM) can efficiently neutralize the toxicity of acrolein, and that eight-membered polymers produced through sequential cycloaddition processes, both within and on the surface of oxidatively stressed cells, are responsible for damaging cellular function.

Our investigations into the biological significance of diaza heterocycles led us to focus on Aβ fibrillation, largely due to the fact that acrolein is produced in the brain tissues of AD patients as a polyamine metabolite during oxidative stress processes. It was speculated that the eight-membered polyamine-acrolein heterocycles (i.e., cSPM or cSPD) may potentially control and/or modulate disease progression. Unlike previous reports suggesting that polyamines promote Aβ40 fibrillation, this study clearly shows that the biologically relevant polyamine-acrolein conjugates inhibit fibrillation and hence cytotoxicity. Thus, the acrolein/polyamine-derived [4+4] cycloaddition process may effectively modulate the oxidative stress processes associated with neuronal diseases.

We initially investigated the effects of cSPM and cSPD on Aβ40 fibrillation. Samples were incubated with 25 × 10⁻⁶ M of the Aβ40 peptide at 37 °C in phosphate buffered saline (PBS)
for 5 d, and fibril formation was evaluated based on the thio-flavin T (ThT) fluorescence assay (Figure 1). Although SPM and acrolein did not show any activity, fibrillization was efficiently inhibited in the presence of cSPD and cSPM at concentrations exceeding $0.5 \times 10^{-6}$ M. Furthermore, one of the diazacyclocotanes, spermine-derived cSPM, effectively suppressed fibrillization for more than a month (Figure 2), indicating that cSPM could steadily inhibit Aβ fibrilization for an extended period of time.

We next examined the activity of Aβ40 samples treated with cSPM using PC12 cells derived from transplantable rat pheochromocytoma (Figure 3). In agreement with previous reports, Aβ40 fibrils are expected to display cytotoxic activity. However, observations indicate that with cSPM treatment in a dose-dependent manner, Aβ40 cytotoxicity was
notably reduced, along with higher cell viability. It should be further noted that this effect was only seen with cSPM concentrations at or greater than 0.5 × 10⁻⁶ M. At cSPM concentrations of 0.1 × 10⁻⁶ M, cytotoxicity was not observed, which is consolidated by the ThT assay results showing fibrillation could not be inhibited in the presence of 0.1 × 10⁻⁶ M cSPM (Figure 1).

The mechanism by which polyamine–acrolein heterocycle cSPM inhibited Aβ40 fibrillation and cytotoxicity was examined by analyzing the molecular sizes of the cSPM-treated Aβ40 peptides using native PAGE/western blotting techniques and an anti-Aβ40 antibody (6E10) (Figure 4a, also see Figure S3a, Supporting Information). This method permitted detection of the soluble monomer at the bottom of the gel (lane 1), whereas the insoluble aggregates, such as the Aβ40 fibrils, remained at the top (lane 5). Intriguingly, a significant quantity of the Aβ40 monomer remained in the Aβ40 samples after treatment with 0.5 × 10⁻⁶ or 1.0 × 10⁻⁶ M cSPM (lanes 3 and 4), although some insoluble species were also detected. The “soluble” oligomeric species, which are considered to be highly toxic to cells,[28–30] were not observed in these gels. The absence of notorious “soluble” aggregates, therefore, supported the significant inhibitory effects of cSPM on cytotoxicity, as observed in Figure 3.

The quantities of the monomeric Aβ40 species present in the cSPM-treated samples in Figure 4a were determined by separating the “insoluble” Aβ40 aggregates from the monomeric peptide by centrifugation (see details in Figure S3a, Supporting Information). The percentages of Aβ40 monomers that remained in the cSPM-treated Aβ40 samples were calculated to be 70%–80% of the mixtures (for samples treated with 0.5 or 1.0 × 10⁻⁶ M cSPM, Figure S3b, Supporting Information). Therefore, it can be concluded that cSPM efficiently suppresses insoluble aggregate formation. As a side note, the insoluble species isolated from the cSPM-treated Aβ40 mixture as the minor product was found to match the cytotoxicity of Aβ40 fibrils (prepared as a control) to PC12 cells (Figure 4b). Together with the TEM images of Figure 5, which show that cSPM could noticeably reduce fibril formation, the collective data suggest that cSPM inhibits cytotoxicity by a mechanism that involves both blocking the formation of highly toxic “soluble” oligomer

Figure 4. a) Native PAGE/western blots of the cSPM-treated Aβ40 peptides. Lane 1: Aβ40 monomer (control), lanes 2–4: Aβ40 was incubated with 0.1 × 10⁻⁶, 0.5 × 10⁻⁶, or 1.0 × 10⁻⁶ M cSPM, respectively, lane 5: Aβ40 fibrils (control). The native marker (Invitorgen) comprising IgM hexamer (1236 kDa), apoferritin (480 kDa), and BSA (66 kDa), which was run separately, was shown as molecular weight marker. b) Comparison of the cytotoxicities of the cSPM-treated Aβ40 mixtures (blue) or insoluble aggregates isolated by centrifugation (red). In assay, PC12 cells (40 000 cells per well) were incubated in the presence of 0.5 × 10⁻⁶ M cSPM-treated Aβ40 for over night, and cell survival was evaluated using the MTT assay. The quantity of the insoluble aggregates was calculated by subtracting the amount of the soluble monomeric peptide (estimated by Micro BCA protein Assay Kit (Thermo Fisher Scientific K. K., Waltham, MA, USA)) from total amount of the Aβ40 peptide used in the experiment.
species and minimizing the formation of toxic “insoluble” Aβ40 fibrils. As a consequence, Aβ40 peptides are expected to be maintained in a monomeric state, thereby reducing cytotoxicity.

Observations also show that cSPM pre-incubated in PBS solution for various time intervals did not induce decomposition or affect the inhibitory activity (see Figure S4, Supporting Information). These eight-membered heterocycles also did not produce any conjugate products involving the lysine groups of model peptides. With these points in mind, it can be strongly speculated that cSPM was therefore the active structure that inhibited fibril formation.

In addition, NMR studies of the Aβ40 peptide titrated using various concentrations of cSPM in PBS revealed chemical shifts at several residues were changed, which includes positions Arg5, His6, Ala21, Ser26, and Asn27 (Figure 6; Figure S5, Supporting Information). Therefore, it could be suggested that these residues may be responsible for suppressing peptide aggregation.

Finally, Aβ40 fibrillation was found to be directly suppressed by in situ generated cSPM (i.e., by simultaneously treating with polyamine and acrolein) by virtue of the facile [4+4] cycloaddition process that occurs in aqueous media (Figure 7). Note that neither polyamine nor acrolein alone inhibited fibrillation, as shown in Figure 1. Given that these heterocycles were produced in vivo as the oxidative metabolites of the polyamines, a unique strategy for neuronal disease treatment may be envisioned.

In summary, we found that 1,5-diazacyclooctanes, the exclusive and biologically relevant products between polyamines and acrolein, inhibited Aβ40 peptide fibrillation and significantly suppressed cytotoxicity. These compounds may inhibit the formation of the highly toxic “soluble” oligomer species while minimizing the toxic “insoluble” Aβ40 fibrillation process. There was no significant difference in inhibitory activity between cSPM and cSPD in Figure 1. The results therefore show that the cyclic 1,5-diazacyclooctane structure is critical to show the activity. The polyamines, SPM and SPD, have different expression level in AD process, but once smoothly reacted with acrolein in/out of the cells under the oxidatively stressed conditions, the corresponding cSPM and cSPD products might similarly inhibit the fibrillation. The results described in this Communication corroborate our discovery that the formal [4+4] cycloaddition reaction is involved in modulating oxidative stress processes associated with neural diseases.

Experimental Section

Inhibition of Amyloid Peptide Aggregation: A stock solution of Aβ40 (1 × 10^{-3} m, solubilized in dimethyl sulfoxide (DMSO)) was diluted to 25 × 10^{-6} m in PBS (pH 7.4) in the presence of various concentrations...
In addition, the effect of DMSO on Aβ aggregation was almost completely inhibited, significant amount of Aβ aggregation was still observed in the presence of 2.5% DMSO, supporting that cSPM could inhibit Aβ aggregation even in the presence of a small amount of DMSO (Figure S2, Supporting Information).

Cytotoxicity Assay: Cell viability was determined using cell proliferation kit (Roche, Basel, Switzerland). PC12 cells (a clonal line of rat pheochromocytoma) were plated in PDL-coated 96-well plates at a density of 40,000 cells per well and grown overnight. The cSPM-treated Aβ samples prepared above was diluted with PBS to the various concentrations (20 µL) and were added to the PC12 cells (in 80 µL of medium). The fluorescence intensity of formazan product at 550 nm was measured by microplate reader (see details in the Supporting Information).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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