The Effect of Cholesterol and Monosialoganglioside (GM1) on the Release and Aggregation of Amyloid β-Peptide from Liposomes Prepared from Brain Membrane-like Lipids*

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In order to investigate the influence of cholesterol (Ch) and monosialoganglioside (GM1) on the release and subsequent aggregation of amyloid β peptide (Aβ(1–40) and Aβ(1–42)), we have examined Aβ peptide model membrane interactions by circular dichroism, turbidity measurements, and transmission electron microscopy (TEM). Model liposomes containing Aβ peptide and a lipid mixture composition similar to that found in the cerebral cortex membranes (CCM-lipid) have been prepared. In all, four Aβ-containing liposomes were investigated: CCM-lipid; liposomes with no GM1 (GM1-free lipid); those with no cholesterol (Ch-free lipid); liposomes with neither cholesterol nor GM1 (Ch-GM1-free lipid). In CCM liposomes, Aβ was rapidly released from membranes to form a well defined fibril structure. However, for the GM1-free lipid, Aβ was first released to yield a fibril structure about the membrane surface, then the membrane became disrupted resulting in the formation of small vesicles. In Ch-free lipid, a fibril structure with a phospholipid membrane-like shadow formed, but this differed from the well defined fibril structure seen for CCM-lipid. In Ch-GM1-free lipid, no fibril structure formed, possibly because of membrane solubilization by Aβ. The absence of fibril structure was noted at physiological extracellular pH (7.4) and also at liposomal/endosomal pH (5.5). Our results suggest a possible role for both Ch and GM1 in the membrane release of Aβ from brain lipid bilayers.

The pathology of Alzheimer’s disease (AD) includes extra- cellular amyloid plaques, intraneuronal neurofibrillary tangles, synaptic loss, and neuronal cell death. The major components of amyloid plaques are the amphiphilic 40 and 42 residue peptides, Aβ(1–40) and Aβ(1–42) (1, 2). Amyloid β-peptide (Aβ) consists of a hydrophilic N-terminal region (residues 1–28) and a hydrophobic C-terminal region (residues 29–40 or 29–42). The hydrophobic part of Aβ is originally part of a transmembrane α-helix of APP anchored in the membrane of several subcellular compartments, including the ER (3). Proteolysis by the enzyme(s) γ-secretase leads to the formation of Aβ within the membrane. Thus, the membrane release of Aβ following this enzyme cleavage should play a pivotal role in subsequent amyloid plaque formation.

Recent studies have shown that the interaction of Aβ and lipids plays an important role in the pathogenesis of AD. For instance, the fibrillogenic properties of Aβ are in part a consequence of the composition of the membrane in which it resides, its peptide sequence, and its mode of assembly within the membrane (4). In terms of membrane composition, Ch and GM1 in neuronal cell membranes are widely accepted to be modulators of membrane-associated Aβ fibrillogenesis and neurotoxicity (5, 6). The formation of GM1-bound Aβ, which is thought to be a seed for the formation of toxic amyloid fiber, depends on the concentration of Ch in model membranes prepared from GM1/Ch/sphingomyelin (SM) (7). Additionally, oligomeric Aβ can promote the release of lipids from astrocytes and neurons by forming Aβ-lipid particles consisting of Ch, phospholipids, and GM1 (8).

It has been suggested that Aβ(1–42) is essential to the early development of AD pathology but is not alone sufficient to promote the formation of mature neuritic plaques unless it is succeeded by the deposition of Aβ(1–40) (9). Compared with Aβ(1–40), Aβ(1–42) has been shown to have a greater potential for aggregation (10). Studies of Aβ-lipid interaction using total brain lipid extract have shown that the peptides interact in different ways: 1) Aβ(1–40) destabilizes model membranes and 2) Aβ(1–42) initially destablizes but then with time proceeds to stabilize the membrane (11). These findings are consistent with a “seeding” hypothesis, in which the aggregates of Aβ(1–42) act as an initiation factor for early plaque formation, which is then followed by the progressive accumulation of Aβ(1–40) in the AD brain. So too, they provide an insight into a mechanism of fibrillogenesis, which is at least in part controlled by the release of Aβ from the membrane.

AD is a disease that involves attack of the central cerebral cortex. To investigate how Ch and the ganglioside GM1 may influence the release of Aβ(1–40) and Aβ(1–42) from cerebral cortex membranes we have prepared and examined some model Aβ-containing liposomes. The liposomes are composed of a lipid-mixture similar in composition to cerebral cortex membranes (CCM-lipid). The liposomes prepared were of four different lipid compositions (see Table I); CCM-lipid, liposomes with no GM1 (GM1-free lipid), liposomes without cholesterol (Ch-free lipid), and liposomes without GM1 and Ch (Ch-GM1-}

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free lipid). Liposomes were prepared by hydrating an organic film composed of a mixture of Aβ and one of the four lipid mixtures outlined in Table I. This method of liposome preparation was chosen to reflect a more natural release process as opposed to a method involving addition of Aβ to preformed liposomes.

The release of Aβ occurs from plasma, endosomal, lysosomal, and Golgi membranes by proteolysis (12–14). All experiments were done at a pH of 7.4 to represent a physiological extracellular pH. However, to model whether or not the more acidic environment of the endosomal pathway is significant, we have carried out a number of our experiments at a pH of 5.5. The common endosomal and lysosomal pH values are thought to be 6.0 and 4.5–5.5, respectively (15, 16); consequently we chose 5.5 to be representative of both organelles. We report here the mode of interaction of Aβ(1–40) and a mixture of Aβ(1–40)/Aβ(1–12) (10/1) with each of the four types of liposome as revealed by CD, turbidity, and negative-staining TEM measurements.

EXPERIMENTAL PROCEDURES

Lyophilized amorphous powders of synthetic Aβ(1–40) and -(1–42) from dimethyl sulfoxide (Me2SO) were obtained from Peptide Institute Inc. (Osaka, Japan), Purities were analyzed by HPLC and amino acid analyses. Egg yolk l-α-phosphatidylcholine (egg PC), egg yolk l-α-phosphatidylethanolamine (egg PE), bovine brain l-α-phosphatidyl-te-serine (PS), bovine brain gangliocerebrosides (Cer), bovine brain sphingomyelin (SM), and Ch were purchased from Sigma-Aldrich Japan. Serine (PS), bovine brain galactocerebroside (Cer), bovine brain sphingomyelin (SM), and Ch were purchased from Sigma-Aldrich Japan. Serine, bovine brain galactocerebroside (Cer), bovine brain sphingomyelin (SM), and Ch were purchased from Sigma-Aldrich Japan and one of the four lipid mixtures outlined in Table I. This method of liposome preparation was chosen to reflect a more natural release process as opposed to a method involving addition of Aβ to preformed liposomes.

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broad negative band is seen around 218 nm, the intensity of which is approximately equal to that of the peak observed for the 7-day-old sample in buffer. But conformational analysis of the spectrum showed 60\% structure and the same spectral pattern after 3, 5, and 7 days. When compared with the CD spectra in buffer, the structure formation was accelerated and more extensive. At pH 5.5, similar CD curves were obtained, though the rate of structure formation was significantly reduced. After 1 day under acidic conditions there was only 36\% structure as compared with 60\% at pH 7.4 (Fig. 1, B-b). On the other hand, the peptide in GM1-free lipid liposomes at pH 7.4 (Fig. 1C), showed a shallow negative band around 210 nm with 25\% helix and 10\% structure, and after 1, 3, and 5 days the negative band had shifted to 216 nm, corresponding to 45\% structure and 10\% helix, indicating that the absence of ganglioside slightly reduces formation of structure, compared with CCM membranes. Interestingly, in Ch-free membrane (Fig. 1D), Aβ-(1–40) had a negative band around 205 nm similar to that of GM1-free liposomes after preparation. Some helical structure (about 15\%) was seen, but in time the band around 205 nm intensified, and a crossing point on the horizontal axis occurred characteristic of structure.
tured. After 3 days, β-structural content reached 50%, indicating, that in time, the absence of either Ch or the absence of GM1 led to a decrease in β-structure formation. For Ch- and GM1-free liposomes (Fig. 1E) at pH 7.4, Aβ-(1–40) showed a broad negative band around 220 nm upon preparation, there was some α-helical structure present. However, in time, the band shifted to 203 nm and became shallower, also the crossing point of the horizontal axis red-shifted to more than 210 nm, indicating decreased β-structure. In fact, the spectra could not be analyzed using the CDPro software (22). Similar CD spectra have been reported in which the spectrum has an atypical absorbance gradually increased from 1 day and reached a maximum at 2 days, very similar CD curves were observed, and the fibril structure was not detected by TEM at either pH (data not shown). Consequently, it would appear that the presence of Ch and GM1 ganglioside is necessary for β-amyloid formation to occur.

CD Spectra of Aβ-(1–40)/Aβ-(1–42) (10:1) in Lipid Bilayers—To investigate a seeding hypothesis in which aggregates of Aβ-(1–42) act as an initiation factor for early plaque formation, we examined changes in conformation of Aβ-(1–40)/(1–42) (10:1, molar ratio) in the four model liposomes at pH 7.4 (Fig. 2) by circular dichroism spectroscopy. Our results show that in CCM liposomes, at a peptide concentration of 40 μM at pH 7.4, solution turbidity did not change at lower peptide concentrations (5–20 μM), the aggregation behavior was dependent on peptide concentration. In conjunction with the CD results, it was concluded that increasing turbidity was attributable to β-structure formation, associated with Aβ aggregation. For 40 μM Aβ in CCM liposomes at pH 7.4, solution turbidity gradually increased over 4 days, then it slightly decreased, but at lower peptide concentrations there was little change in turbidity (Fig. 3B). With the results of CD and TEM experiments in mind, little or no change in turbidity is due to an aggregation of Aβ-(1–40) to form a β-structure. Similar turbidity changes in CCM were obtained at pH 5.5 (data not shown). In the GM1-free liposomes at pH 7.4, solution turbidity did not change at the examined Aβ concentrations except for 40 μM peptide, where a slight decrease occurred 1 day after preparation and was followed by a gradual increase (Fig. 3C). Little or no change in turbidity was consistent with the CD and TEM results, which showed the formation of aggregated peptide-lipid particles (to be described below). Interestingly, in Ch-GM1-free lipid liposomes at pH 7.4, turbidities for 20 and 40 μM peptide decreased. For 40 μM peptide an especially dramatic decrease in turbidity occurred over the first day (Fig. 3, E-a). Similar changes in turbidity were observed at pH 5.5 (Fig. 3,
E-b). The decrease in turbidity indicated liposomes were solubilized by Aβ-(1–40), to form small peptide-lipid particles. The TEM image for this solution showed the liposomes became smaller with time and showed no fibril structure. Although a similar decrease in turbidity was observed for the Ch-free liposome solution (Fig. 3D), this was independent of peptide concentration; the turbidity of 20 μM Aβ was much larger than that of 40 μM. This also indicated that liposomes were solubilized and led to the formation of small particles.

Turbidity of Aβ(1–40)/Aβ(1–42) (10:1) was measured in CCM lipid and ChGM1-free lipid at pH 7.4 (data not shown). Initial turbidity did not depend on peptide concentration and did not change dramatically with time, which indicated the membrane solubilization did not occur as it did for Aβ-(1–40) in Ch-free and Ch-GM1-free membranes.

Studies by Transmission Electron Microscopy—To observe changes with time in the morphological characteristics of Aβ-(1–40) and Aβ-(1–40)/Aβ(1–42) (10:1) both in buffer and in the liposomes we made measurements by TEM. Liposomes were prepared from a mixture of lipids (1 mM) and peptide (50 μM) and then examined over a period of 1–10 days under the electron microscope. The TEM samples were negatively stained. For Aβ-(1–40), after 1 day in buffer solution at pH 7.4, an indistinct fibril structure was observed, which after 10 days became better defined (Fig. 4A). In CCM lipid membrane at pH 7.4, a distinct fibril structure was observed, with a dark phospholipid membrane-like shadow after 1 day (Fig. 4B-a, i). After 10 days the same clear fibril structure seen in saline buffer was observed (Fig 4B-a, iii). At pH 5.5, lots of short filaments were visible after 1 day and clear long fibrils were observed after 3 days, indicating that the growth of fibril structure is slower than that at pH 7.4 (Fig 4B-b). This is consistent with the slower rate of β-structure formation seen by CD after 1 day at pH 5.5 relative to that at pH 7.4. In GM1-free membranes (Fig. 4C) at pH 7.4 after 1 day, long fibril structures were present along the surface of vesicles, which were probably large aggregated/fused liposomes. After 3 days, small vesicles emerged around the large aggregate liposomes, also some fibrils were still apparent along their surface. After 10 days we observed aggregates of small vesicles (several ten-fold nanometers in diameter). These results indicate that Aβ can disrupt the large liposomes into smaller vesicles, presumably by the formation of lipid-peptide complexes. After 1 day in the Ch-free membrane (Fig. 4D) at pH 7.4, a few relatively long fibrils were observed around the large liposomes. After 4 days, numerous small filament-like structures, probably lipid-peptide complexes, were present around the liposomes. Interestingly, after 10 days, thicker and longer fibrils (several ten-fold nanometers in diameter) were observed around the liposomes, which were different from the fibril structure observed in the buffer and CCM liposomes.

These results indicate that although the Ch-free membrane first releases some Aβ to create fibrils, in time, the membranes were slowly solubilized by Aβ to make short and thin fibrils and...
eventually thicker and longer fibers. In Ch-GM1-free membranes at pH 7.4 and 5.5 (Fig. 4, E-a and -b) fibrils were not observed, liposomes of various shapes and sizes were present, and with time an increase in the number of smaller vesicles occurred.

Images of Aβ-(1–40)/Aβ-(1–42) (10:1) in CCM (Fig. 4F) were very similar to those for Aβ-(1–40) (Fig. 4B), showing indistinct fibril structures after 1 day and extensive well defined ones after 10 days (Fig. 4F). Similar images (not shown) were observed for GM1-free membranes. Interestingly, in Ch-GM1-
free membranes, both fibrils and spherical liposomes were present after 1 day, although with time the number of fibrils increased and the spherical liposomes disappeared (Fig 4G). Similar images (data not shown) were observed for the Ch-free membranes.

**DISCUSSION**

Because Aβ peptides are generated by the partial processing of the transmembrane α-helix of APP anchored in the brain membrane, their release from the membrane must play an important role in their subsequent aggregation and precipitation. Thus, to investigate how membrane lipids participate in the formation of fibril structure, we monitored the release of Aβ peptide from Aβ peptide-model membranes. Liposomes were prepared from Aβ and a lipid-mixture similar in composition to that of cerebral cortex membranes with and without ganglioside and/or cholesterol.

Our CD studies have shown that when a 50 μM solution of Aβ(1–40) in HFIP solution is hydrated in buffer solution, it undergoes a transition from random coil to β-structure over a period of 3 days (Fig. 1A). This is consistent with the turbidity measurements. Over 24 h, the turbidity did not change, but it rapidly increased from 1 to 3 days, and only moderately after 3 days (Fig. 3A). From these results we propose that hydration of monomeric Aβ(1–40) in organic solvent caused a slow change in conformation from random coil to β-structure. This β-struc-
ture then serves as a seed in the rapid formation of an extensive β-sheet structure (28). TEM measurements supported the presence of an extended fibril formation just 3 days after peptide hydration.

In CCM membranes at physiological pH (7.4), Aβ-(1–40) was mainly random coil after preparation, although some α-helical and β-sheet content was present (Fig. 1B). After 1 day, mainly β-structure had formed which persisted for 10 days. The turbidity increased moderately only for high concentrations of Aβ-(1–40) (Fig. 3B-a). TEM images of fibrils seen after 1 day (Fig. 4B-a, i) were clearer than those observed for the buffer solution. At pH 5.5 (Fig. 4B-b, i) the presence of fibrils after 1 day is uncertain, although after 3 days an extensive fibril formation is apparent (Fig. 4B-b, ii). For the CCM liposomes at endosomal pH the rate of formation of β-structure and fibrils is definitely slower than at physiological pH; however, the final resulting fibril structures appear the same. That Aβ fibril formation is pH-dependent has been reported in the literature (45).

From the CCM liposomes Aβ-(1–40) was released rapidly, and resulted in the formation of a fibril structure. In contrast, in GM1-free liposomes, Aβ-(1–40) consisted of a mixture of α-helix and β-structure and with time the proportion of β-structure increased. However, a slight increase in turbidity was observed for 40 μM Aβ. Its TEM image showed at first an incomplete short fibril structure around the lipid surface, and in time, small vesicles began to emerge, until finally only aggregates of small vesicles were visible (Fig. 4C). Apparently, Aβ was able to solubilize the ganglioside-deficient membrane into small vesicles.

In Ch-free Lipid liposomes, a similar change from α-helix to β-structure was observed. The turbidity decreased drastically for 20 μM Aβ, but not so for 40 μM. However, there were few signs of fibril structure after 1 day; instead spherical liposomes were visible (Fig. 4D, i). From 3 to 14 days, a gradual thickening and elongation of the thin, short fibrils around the spherical vesicles took place (Fig. 4, D, ii and iii). These fibrils were different in appearance to those observed in the buffer and CCM membranes. We propose these were peptide-phospholipid membrane complexes, because we have shown previously that highly hydrophobic peptides can form nanotubular fiber structures (29–31). Ch may assist in fibril formation, by promoting the release of Aβ-(1–40) from natural membranes. Interestingly, in both GM1- and Ch-free liposomes, the CD spectra show a shallow minimum around 223 nm (Fig. 1D), which was not seen for the CCM and GM1-free liposomes. Moreover, the TEM showed liposomes of various shapes and sizes, but no fibril structure was seen. These phenomena were the same at neutral and acidic pH membrane solutions. This suggests that the coexistence of Ch and ganglioside in the CCM membrane has a crucial role in the release of Aβ-(1–40) from the membrane and the subsequent formation of fibril structures.

A recent study has shown that in different lipid membranes Aβ can follow two pathways of assembly: pathway 1) the formation of fibril structure in the presence of acidic lipids; and pathway 2) the formation of small aggregates (but no fibril structures) in the presence of neutral lipids (32).

This study shows GM1-free liposomes promote the formation of β-structure and small peptide-lipid vesicles, several ten-fold Angstrom in diameter. This process may take pathway 2; the absence of GM1 leads to the decrease in the acidity of the membranes, resulting in the elimination of Aβ-fibril formation. However, membrane disruption still occurs via the expansion of aggregated Aβ through the bilayers, resulting in the solubilization of membranes to form small peptide-lipid vesicles. Matsuzaki and Horikiri (33) reported that Aβ has a high affinity for GM1 ganglioside in the bilayer and is able to form a β-sheet structure. It has been reported that the tight binding of Aβ is to the sialic acid group of the GM1 (34). In the presence of GM1, Aβ probably follows pathway 1, a conformational transition from α-helix to a β-structure leading to fibril formation. However, tight binding of the peptide to GM1 may prevent its release from the membrane, resulting in accumulation of peptide and formation of a β-sheet scaffold structure. This may be the critical nucleus for fibril formation. After nucleation, fibril growth through the lipid bilayer results in destabilization of the membrane, leading to amyloid deposition or the formation of lipid particles (to be described below).

Ch promotes fibril structure formation as we observed by TEM; in the absence of Ch, a well defined fibril structure was not visible even after a few days. Interestingly, in the absence of Ch or GM1 Aβ does form a β-structure, so the formation of a β-structure does not necessarily lead to fibril formation. An increase in Ch in the membrane results in increased membrane stiffness and a decrease in membrane fluidity. Increased Ch content inhibits the insertion of Aβ into the membrane, resulting in an increase in Aβ concentration at the membrane surface and concomitant enhancement in the rate of Aβ fibrillogenesis (5). Therefore, the absence of Ch in the membrane will increase its fluidity, and thus facilitate the insertion of the hydrophobic part of Aβ into the membrane (25). In Ch-free liposomes, the accumulation of Aβ into the membrane leads to its solubilization, resulting in the slow formation of thick fibril structures.

In the absence of both Ch and GM1, the characteristic CD pattern of β-structure with a negative at around 116 nm was not seen, and no fibril structure was observed by TEM. This suggests the coexistence of Ch and GM1 in the CCM membrane promote fibril formation. A strong increase in turbidity at 40 μM Aβ was also observed. All of these results suggest that the Ch-GM1-free membrane was solubilized by Aβ (1–40). Yanagisawa et al. (6) have shown that GM1 ganglioside-bound amyloid β-proteins are a possible form of preamyloid in AD. They also reported that oligomeric Aβ can promote the release of lipid from neurons to form Aβ-lipid particles consisting of Ch, GM1, phospholipid, and Aβ. Recent model membrane studies using liposomes consisting of GM1, Ch, and sphingomyelin showed that an increase in GM1 as well as Ch changes the binding capacity of Aβ (7, 35). Our data indicate that GM1 and Ch strongly participate in the release of Aβ from the membrane and therefore are instrumental in amyloid precipitation.

It has been suggested that sphingolipids and cholesterol may exist as phase-separated “rafs” in sphingolipid and cholesterol-rich membranes such as the plasma membrane (36). The partial liquid-ordered rafts can be visualized as floating within the predominantly liquid crystalline “sea” of the lipid bilayer. Interestingly, it was proposed that the raft could be the site for the proteolytic processing of Alzheimer’s amyloid precursor protein (APP) (37). Recently, we showed that elevation of sphingolipid and Ch cause decreased membrane fluidity and resulted in lipid-protein separations into liposomes containing α-helical transmembrane peptides (38). The proteolytic cleavage of APP to yield Aβ performed by both β-secretase and γ-secretase present in the raft may in fact involve the release of Aβ-lipid particles consisting of Ch, GM1, and phospholipid (8). However, in Ch- and GM1-free membranes, which are more fluid, Aβ is able to stay in the membrane, probably by insertion of its hydrophobic part into the lipid bilayer. The accumulation of Aβ in the membrane may result in its solubilization and eventual disruption into small vesicles.

The CCM consists of about 10% (w/w) plasmalogen (24), but the instability of this component under acidic conditions prevented its use in this study. We note, however, that it has been
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reported in the literature that levels of plasminogen in AD CCM are reduced (39, 40).

Aβ(1–42) has been recognized to be the more amyloidogenic component in plaques, since it has a greater propensity to form β-structure than Aβ(1–40), a requirement for amyloid fibril formation. Consequently, aggregates of Aβ(1–42) may act as an initiation factor for early plaque formation (10). In the present study, Aβ(1–40)/(1–42) (10:1, molar ratio) formed β-structure more readily than Aβ(1–40) in all classes of liposome. Especially, in Ch-GM1-free membranes, where Aβ(1–40) forms no β-structure, the mixture of Aβ(1–40)/(1–42) caused the gradual conversion of a predominantly α-helical structure into a mainly β-structure. Therefore, the presence of a small amount of Aβ(1–42) can induce the formation of β-structure in Aβ(1–40) and confirms the “seeding” hypothesis. Aβ(1–42) is able to promote the formation of β-structure that accompanies the formation of fibrils.

Recent studies have demonstrated that amyloid plaque formation may be initiated in the plasma membrane and that deposits were associated with the extracellular leaflet of the plasma membrane (41, 42). Moreover, Aβ-amyloid peptides are generated from various intracellular compartments, including the endoplasmic reticulum, the Golgi apparatus, lysosomes, and endosomes. The present model studies are carried out at the extracellular and lysosomal/endosomal pH values of 7.4 and 5.5. The results for the CCM liposomes indicate a kinetic pH-dependence of fibril formation, but it is not clear whether the release of Aβ(1–40) is also pH-dependent. The results seen for the Ch-GM1-free liposomes are the same in both pH environments; fibril formation does not occur. This definitely indicates that lipid bilayer composition plays an important role in the release of Aβ and might suggest that this release is much less dependent on the pH of the surrounding cytosol.

It has been shown that ganglioside and Ch participate in the mechanism of amyloid deposition in the presence of total brain lipid extract (5, 11, 34). However, until now there has been no report in the literature on the behavior of Aβ in membranes free of both Ch and GM1. We have shown that Ch and GM1 play an important role in the release of Aβ from liposome membranes designed to model cerebral cortex membranes, where fibril structure formation is known to be at its highest. In natural brain membranes, the Aβ generated from the processing of APP may be easily released from the membrane to play its correct biological role. However, the change of lipid composition in membranes by aging or other biological processes induces Aβ accumulation in membranes, this leads to formation of amyloid fibers or lipid-peptide particles, which are released into the cytosol, resulting in amyloid precipitation or cytotoxicity.

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