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Dendrimers-drug conjugates for tailored intracellular drug release based on glutathione levels

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Abstract

N-Acetyl-L-Cysteine (NAC) is an anti-oxidant and anti-inflammatory agent with significant potential in clinical applications including stroke and neuroinflammation. The drug shows high plasma binding upon IV administration, requiring high doses and associated side effects. Through the use of an appropriate delivery vehicle, the stability and efficacy of NAC can be significantly improved. Dendrimers are an emerging class of nanoscale drug delivery vehicles, which enable high drug payloads and intracellular delivery. Poly(amidoamine) dendrimer-NAC conjugates having cleavable disulfide linkages are designed for intracellular delivery based on glutathione levels. We have successfully synthesized two conjugates of with a cationic G4-NH₂ and an anionic G3.5-COOH PAMAM dendrimers with NAC payloads of 16 and 18 per dendrimer, respectively, as confirmed by ¹H-NMR and MALDI-TOF analysis. NAC release kinetics of the conjugates at intracellular and extracellular Glutathione (GSH) concentrations were evaluated by reverse phase HPLC (RP-HPLC) analysis, and ~70% of NAC payload was released within one hour at *intracellular* GSH concentrations (~10 mM), whereas negligible NAC release was observed at *extracellular* GSH levels (2 μM). FITC-labeled conjugates showed that they enter cells rapidly and localize in the cytoplasm of lipopolysaccharide (LPS)-activated microglial cells (the target cells *in vivo*). The efficacies of dendrimer-NAC conjugates in activated microglial cells was confirmed by measuring the nitrite inhibition in the cell culture medium, which is an indication of the anti-oxidative property of the drug.

Both G4-NH₂ and G3.5-COOH conjugates showed more significantly better nitrite inhibition both at 24 and 72 hours compared to free NAC, by as much as a factor of 16. The results indicate that PAMAM dendrimer conjugates produce a higher local NAC concentration inside the cells, with GSH-sensitive disulfide linker enabling efficient and rapid cellular release of the drug.

Introduction

N-acetyl cysteine (NAC), is a clinically important, antioxidant, anti-apoptotic, and anti-inflammatory drug used in the treatment of neuroinflammation, AIDS, colon cancer, and detoxification of heavy metals (*e.g.* lead, mercury, arsenic) (1–6). NAC has been extensively

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studied as both a therapeutic agent and direct Cysteine precursor (7). In the treatment of neuroinflammation, it acts at multiple neuroprotective sites, and has recently been demonstrated to attenuate amniotic and placental cytokine responses after maternal infection induced by lipopolysaccharide (LPS) (8), and to restore the maternal fetal oxidative balance and reduce fetal death and preterm birth (9,10). Further, higher dose of NAC remains a primary treatment for acetaminophen overdose and exposure to toxic chemicals and is routinely used in hospitals (11–14). However, the use of NAC requires higher and repeated dosing. This is due to the poor bioavailability and blood stability, caused by the presence of free sulfhydryl groups in NAC which are capable of spontaneous oxidation, and forming disulfide bonds with plasma proteins (15). Early pharmacokinetic studies have demonstrated low oral bioavailability of NAC between 6–10%, which were attributed to low blood concentrations of NAC (16,17). The need for high doses can lead to cytotoxicity and side effects, including increased blood pressure (18). NAC is one of the very few drugs approved for treating neuroinflammation in perinatal applications, where side effects can be very critical.

Through the design of appropriate dendrimer-NAC conjugates can improve the stability and bioavailability, at the same time enable intracellular release. These are especially important in our eventual interest in perinatal and neonatal applications of dendrimers and NAC. The unique design of conjugates involves linking of the NAC via disulfide bonds to spacer molecules attached to dendrimers. The resulting structure of the conjugates described here, achieves two major objectives to ensure efficacy; (a) it may restrict the protein binding of NAC as the free sulfhydryl groups are involved in disulfide linkages, (b) it may enable higher intracellular levels of NAC, and better release of NAC from the conjugate, resulting from disulfide linkages that are cleaved in presence of intracellular glutathione (GSH). The results on *in-vitro* release and the cellular efficacy towards reducing neuroinflammation in activated microglial cells shows the improved efficacy of the conjugates.

Over the past few decades, polymeric carriers have been extensively explored for controlled delivery of drugs intracellularly and to targeted tissues (19). Dendrimers are emerging as a viable class of polymeric vehicles (~5–15 nm), because of the large density of reactive functional groups and a well-defined structure and monodispersity (20,21). This enables a high drug payload, but the steric hindrance at the dendrimer surface can make drug release a challenge when ester or amide linkers are used, especially at higher generations (22). Active molecules could be encapsulated (23), complexed (24), or covalently linked (25) to the polymeric carrier. The polymer can improve the solubility, stability, and blood circulation times. Despite several significant achievements of the polymeric conjugates, clinical applications still remain elusive, partly due to the issues of drug release over an appropriate time interval. Common approaches in conjugate design involve the use of ester or amide linkers, which are cleaved hydrolytically or enzymatically (26). For practical applications in drug delivery, increasing the drug payload and engineering the drug release at the appropriate tissue are two key aspects in the design of polymer conjugates. For intravenous applications, it is highly desirable to design a linker that is stable during circulation, but enables drug release when they reach the target site. There is a need to design the efficient polymer conjugates having the cleavable bonds or linkers, with high drug payloads, and appropriate release profiles.

Recently, specific chemical reactions, such as the disulfide reduction, have emerged as alternative mechanisms for drug release (27,28). Polymeric delivery systems, offer an avenue for GSH responsive targeted delivery of drugs to tumor tissue (29,30). Various carriers such as gold nanoparticles (31), gold nanorods (32), mesoporous silica nanorods (27), nanoparticle inhibited β -galactosidase (33), poly (2-dimethylaminoethyl methacrylate) (PDMAEMA) (34), carbon nanotubes for siRNA delivery (28), poly (β -amino ester) (35), gelatin nanoparticles (36), methyl acryloylglycylglycine 4-nitrophenyl ester for DNA delivery (37), have been used in this regard with reductively cleavable disulfide spacers. Furthermore

disulfide bonds have been incorporated in the synthesis of cleavable delivery systems for plasmid DNA (38), antisense oligonucleotides (39), peptide nucleic acids (40), toxins (41), and anticancer drugs (42). Therefore, in the present investigation we have combined a dendrimer-based delivery system with disulfide chemistry, to develop a GSH-responsive release system with a high drug payload. The disulfide bonds are easily cleavable and hence the drug release is not compromised. The glutathione (GSH)-mediated release of biomolecules from monolayer protected gold nanoparticle surfaces and manipulation of their bioactivities *in vitro* has been demonstrated (31). GSH is the most abundant thiol species in the cytoplasm and the major reducing agent in biochemical processes, providing a potential *in situ* releasing source in living cells. The intracellular GSH concentration (1–10 mM) is substantially higher than extracellular levels (0.002 mM in plasma) (31). More importantly, the GSH levels in cancer tissues can be many-fold higher than those in normal tissues (30,31). Therefore, a GSH responsive linker will limit plasma release and can promote targeted release.

Experimental Procedure

The experimental protocols, and the characterization methods, and the NMR and MALDI spectra, for the various compounds discussed in the paper are provided as part of supporting information (R-I to R-XI).

Stability of Conjugates

Stability of NAC, GSH, GSH and NAC when they are present together were evaluated as controls. The NAC and GSH concentrations used for stability studies were 0.2 mg/mL and 3 mg/mL (10 mM) respectively. The concentrations of GSH and NAC in these studies were chosen in relevance to the conjugate stability studies. Stability of conjugates was analyzed in physiological pH (PBS buffer) and in the presence of two different GSH concentrations (10 mM and 2 mM). The stability studies were carried out for a period of 17 hours at 37 °C. The conjugates were dissolved at 1 mg/mL concentrations in 1 mL of PBS solution and required amount of GSH was added to trigger disulfide exchange reactions, resulting in the release of NAC from the conjugate. At specific time intervals, 30 μ L samples were withdrawn and analyzed with HPLC. All experiments were run in triplicate for statistical analysis.

Flow Cytometry Analysis

Immortalized mouse microglial cells (BV-2 cells obtained by courtesy of Dr. Kuhn, Wayne State University) (passage 16) were grown overnight in 6-well plate using DMEM cell culture medium supplemented with 10% FBS and 1% penicillin-streptomycin. When the cells were 70% confluent, they were treated with FITC-labeled dendrimer-NH- conjugated N-Acetyl-L-cysteine Fluorescein isothiocyanate-PAMAM-NH-CO-Propionyl-S-S-N-Acetyl-Cysteine (FITC PAMAM-NH-CO-Pr-S-S-NAC compound 2) (equivalent to 10 μ g/mL of FITC-PAMAM-NH-CO-Pr-S-S-NAC) for 15, 60 and 120 min. The cells were washed with phosphate buffered saline (PBS, pH = 7.4), trypsinized and centrifuged at 1500 rpm for 5 min to obtain a cell pellet. The cells were then rinsed with PBS buffer, spun down three times, and resuspended in 1% formaldehyde, and analyzed using a flow cytometer (FACS caliber, Becton Dickinson) by counting 10,000 events. The mean fluorescence intensity of cells was calculated using the histogram plot.

Confocal Laser Scanning Microscopy

The procedure for cell culture and drug treatment was the same as described in previous section. After treating BV-2 cells for 2 hours with FITC PAMAM-NH-CO-Pr-S-S-NAC conjugate (equivalent to 10 μ g/mL of FITC PAMAM-NH-CO-Pr-S-S-NAC), the cells were washed with PBS three times and fixed with 4% para-formaldehyde for 20 min. Images were captured under

the confocal microscope (Zeiss LSM 310) using a magnification of 630 ×. The emission and excitation wavelengths were 488 and 518 nm for FITC.

Detection of Nitrite Production

The presence of L-PS induces nitrite production, and the subsequent suppression of this by the dendrimer conjugates is used to assess the efficacy. BV-2 cells (passage 16) were seeded in 24 well plate at 10^5 /ml/well and incubated for 24 hours. The medium was removed and 500 μ L of fresh serum free medium was added. The cells were exposed to 100 ng/mL of lipopolysaccharide (LPS) and various concentrations of dendrimer conjugates for 3 hours. The medium was removed again and 500 μ L of fresh, serum free medium containing 100 ng/mL of LPS was added. The cells were incubated for 24 and 72 hours, and the culture medium was removed for analysis. Control treatments with various concentrations of free NAC, positive controls with LPS induction and no treatment, and negative controls without any LPS induction were also studied.

Accumulation of nitrite in the culture medium was used as a measure of NO formation. The nitrite concentration was determined by using the Griess reagent system (Cayman) that uses sulfanilamide and N-(1-Naphthyl)-ethylene diamine. In brief, 100 μ L of supernatant from BV-2 cells exposed to different treatments was incubated with 50 μ L of Griess reagent 1 (sulfanilamide) and 50 μ L of Griess reagent 2 (N-(1-Naphthyl)-ethylenediamine) for 10 min at room temperature. The absorbance at 540 nm was then measured, and nitrite concentration was determined using a curve calibrated with nitrite standards.

Intracellular GSH measurement

Levels of intracellular reduced glutathione (GSH) was assessed spectrofluorimetrically by monochlobimane staining (13). Briefly, the procedure for culture and drug treatment was the same as described in previous section. Cells seeded in collagen I coated 96-well plates were washed once with PBS and were incubated with 50 μ M monochlobimane diluted in phenol red free medium. The fluorescence intensity was measured after 15 min at 37°C. Excitation and emission wavelengths were 380 nm and 485 nm, respectively. Intracellular GSH reduced rate was calculated according to formula: [Reduced rate (%) = (fluorescence intensity of EMEM control - fluorescence intensity of treatment group)/ fluorescence intensity of EMEM control $\times$ 100%].

Results and Discussion

This therapeutic efficacy of polymer-conjugates can be enhanced, and side effects reduced, if intracellular drug release can be enhanced. This is especially true in neonatal applications of NAC, where high doses of NAC are used. To attain this objective, we have developed GSH-responsive dendrimer NAC conjugates incorporating a connecting disulfide spacer. Use of the disulfide spacer demonstrated extracellular stability with rapid degradation once internalized in cells, releasing the free NAC. In the present investigation, we have successfully synthesized and evaluated two dendrimer conjugates, a cationic PAMAM-NH-CO-Pr-S-S-NAC and an anionic G3.5-CO-Glutathione-S-S-NAC (G3.5-CO-GS-S-NAC) conjugate, for the first time in dendrimers with disulfide bond between drug and dendrimer through different spacer. Drug will be released at a rate dependent on GSH concentration.

PAMAM-NH-CO-Pr-S-S-NAC conjugate synthesis (1)

To conjugate the NAC to dendrimers, the linker SPDP was appended to the dendrimer with the thiopyridine termination. The NAC was covalently attached to the PDP linked dendrimer by formation of disulfide bonds. Synthesis of N-succinimidyl 3-(2-pyridyldithio)- propionate (SPDP) was performed by a two-step procedure (Scheme-1). First 3-mercaptopropionic acid

was reacted by thiol-disulphide exchange with 2, 2'-dipyridyl disulphide to give 2-carboxyethyl 2-pyridyl disulphide (Scheme-1, Supporting information R-V). To facilitate linking of amine terminated dendrimers to SPDP, the succinimide group was appended on SPDP to obtain N-succinimidyl 3-(2-pyridyldithio) propionate (Scheme-2, Supporting information, R-VI). by esterification with N-hydroxysuccinimide by using NN'-dicyclohexylcarbodi-imide. To introduce sulfhydryl-reactive groups, PAMAM-NH₂ dendrimers were reacted with the heterobifunctional cross-linker SPDP (Scheme-2, Supporting information, R-VI). The N-succinimidyl activated ester of SPDP couples to the terminal primary amines to yield amide-linked 2-pyridyldithiopropionyl (PDP) groups (Scheme-2). After reaction with SPDP, PAMAM-NH-PDP was analyzed using RP-HPLC (data not shown) to determine the extent to which SPDP had reacted with dendrimers.

The samples were compared to unmodified PAMAM-NH₂ dendrimers. Samples were initially run on a linear gradient from 100: 0 H₂O(0.1 wt % TFA)/acetonitrile to 10:90 H₂O (0.1 wt % TFA)/acetonitrile over 32 min. During this gradient, PAMAM-NH₂ was eluted after (13.1) compared to the modified PAMAM-NH-PDP dendrimer (data not shown). The increased retention time is in line with addition of hydrophobic PDP groups. The slight broadening of the peaks and appearance of shoulder peaks present for both PAMAM dendrimers and PAMAM-NH-PDP might reflect structural defects that occurred during synthesis of the dendrimer, for example, by incomplete alkylation of the primary amines or intramolecular cyclization. The absence of amine-terminated dendrimer in the pyridyl disulfide-modified dendrimers, indicates the completion of the reaction as reflected from the HPLC analysis. The PAMAM-NH-PDP so obtained was reacted with water soluble NAC to get desired conjugate. The linking of NAC to dendrimer via formation of disulfide bond was confirmed by HPLC, NMR and MALDI-TOF (Table-1, Supporting information, R-VII). The NMR and the MALDI data for the drug payload agree very well with each other, as summarized in Table 1. The HPLC chromatogram reflected decreased retention time (Figure-2B) (15.0min) with the addition of hydrophilic groups. The shift in retention times for the dendrimers conjugates confirms the conjugation with NAC, further the shift to higher retention times indicates the hydrophobic nature imparted due to the spacer molecules and the NAC. The absence of the peaks corresponding to NAC and NAC-NAC, and SPDP in the chromatogram for the conjugates confirms the purity of the compound synthesized. The chromatogram of PAMAM-NH-CO-Pr-S-S-NAC conjugate showed presence of very small fraction of NAC-NAC as indicated by the slight hump at 8.2 min. Further, appearance of methyl groups in NMR at 1.94 ppm confirms the formation of disulfide bond between the PAMAM-NH-PDP and NAC. The attachment of multiple copies of NAC to PAMAM-NH-PDP dendrimers was determined by MALDI-TOF. Analysis of the unmodified G4 dendrimer gave a broad M⁺ peak at 14.1 kDa, (Figure-1A) which closely corresponds to the theoretical molecular mass of the dendrimer 14.2 kDa. Coupling of the G4 terminal amine groups with NAC resulted in a shift in the major peak to 18.3 kDa (Figure-1B, Table-1, Supporting information, R-VII). Each thiopropanoyl NAC group has a molecular mass of 250 Da. Therefore, these data indicate an average of 16 NAC molecules per dendrimer molecule (16 thiopropanoyl NAC groups per dendrimer molecule containing 64 amine terminal groups, $n = 3$; number of independent experiments, Table-1, Supporting information, R-VII). PAMAM-NH-CO-Pr-S-S-NAC conjugate was tagged with fluorescent dye FITC (Scheme-3) for cell uptake study (data not shown). The drug payloads in the conjugates have been kept moderate, in order to enable high solubilities of the conjugate for *in vivo* experiments.

PAMAM - CO -GS-S-NAC conjugate synthesis

S-(2-thiopyridyl) glutathione, was prepared from the reaction of 2, 2'-dithiodipyridine in excess and the corresponding peptide in a mixture of methanol and water at room temperature (Scheme-4). Upon completion of the reaction, methanol was removed in vacuo and the residue

was washed with dichloromethane. The aqueous solution was subjected to reverse phase (RP) HPLC purification, and lyophilization of the eluent gave the pure product as a white solid (Supporting information, R-IX). This compound was reacted with NAC in PBS in pH = 7.4 to get desired Glutathione-N-Acetyl Cysteine (GS-S-NAC) (Scheme-4) intermediate and purified. The formation of disulfide bond was confirmed by NMR and ESI-MS (Table-1, Supporting information, R-X). Appearance of methyl groups in NMR at 1.90 ppm indicates the formation of disulfide bond between the GSH and NAC. To introduce the GS-S-NAC, PAMAM-COOH was reacted with GS-S-NAC in the presence of PyBop/DIEA to give desired PAMAM-CO-GS-S-NAC conjugate (Scheme-4, Supporting information, R-XI). Introduction of GS-S-NAC was confirmed HPLC, NMR, and MALD. The same type of MALDI analysis yielded approximately 19.7kDa (Figure-3B, Supporting information, R-X) (18 GS-S-NAC groups for the PAMAM-COOH dendrimers). The number of GS-S-NAC groups was also determined via NMR analysis and the appearance of methyl protons at 1.70, 1.92 ppm (Supporting information, R-XI) indicates the formation of GS-NAC conjugate with dendrimer. The NMR and the MALDI data for the drug payload agree very well with each other, as summarized in Table 1. The yields of PAMAM-conjugates are high and reproducible.

Release Studies

The release of NAC from the conjugates was investigated in presence of GSH at intracellular and extracellular concentrations (31). It was assumed that the release of NAC would occur by the disulfide exchange reactions. GSH and its oxidized form (GSSG) are responsible for forming the intracellular redox buffer. Intracellularly, GSH takes the role of attacking thiolate moiety and gets oxidized at the process while cleaving the existing disulfide bonds. Disulfide exchange reactions do not change the total number of disulfide bonds but rather shuffle the species forming them. In the present study the release of NAC from the conjugates by disulfide exchange reactions was confirmed by the HPLC analysis and is discussed in detail in the following sections.

Free NAC had an elution time of 4.7 minutes (Figure-5, a) whereas GSH eluted at 3.8 minutes (Figure- 5, c). Oxidized forms of NAC and GSH were also analyzed by HPLC and oxidized form of NAC eluted (NAC-NAC) at 8.2 minutes (Figure- 5, b) while oxidized GSH (GSSG) eluted at 3.9 minutes (Figure -5, d). GSSG peak was very close to GSH peak and when both were injected together GSSG appeared as a shoulder on the GSH peak (Figure-5, d). On the other hand, NAC-NAC is more hydrophobic than NAC as indicated by the higher elution times for the former than NAC in the chromatogram (Figure-5, b) Hydrophilicity of NAC can be associated with its thiol group and when this group is occupied the molecule is rendered more hydrophobic, as suggested by significant increase in its retention time when NAC-NAC (Figure-5, b) was formed. Similar shift to higher retention was observed for GSSG as indicated by the appearance of shoulder on the peak seen for retention of GSH (Figure 5, d). However, this shift in retention time of GSSG is not as significant (Figure-5, d) as seen for NAC-NAC (Figure 5, b). Retention time of GS-S-NAC (Figure 5, e) was (5.3 min) which was longer than both GSH (3.8 min) and NAC (4.7 min). This suggests that occupation of thiol groups reduces the hydrophilicity of both NAC and GSH.

Stability analysis of free NAC and free GSH suggests that both GSH and NAC go through slow oxidation and form their dimers (NAC-NAC and GSSG) by disulfide bond formation when dissolved in PBS. The rate of disulfide bond formation was relatively slow at 25% of NAC and GSH being converted to their oxidized form over 17 hours. It was determined that in addition to their dimers, when NAC and GSH were present together; they formed GS-S-NAC as well. The formation rates of GS-S-NAC were in agreement with the oxidation rates determined for both NAC and GSH separately. When NAC and GSH were present together where GSH was in excess, no detectable NAC-NAC was formed. The conjugates were also

analyzed in the absence of GSH to verify the stability of the disulfide linkage at physiological pH. When the conjugates were placed in PBS buffer and analyzed for 17 hours, both conjugates were stable and did not release any of the NAC they carried. The stability of the disulfide linkage shows that they are capable of carrying their payload without any release due to instability in aqueous media at physiological pH. The extent of release of drug from both dendrimer-NAC conjugates were also analyzed at plasma and intracellular GSH concentrations. The conjugates and the GSH in required amounts were added to PBS solution and the solution was analyzed at various time intervals by HPLC for up to 17 hours. UV absorbance peak areas were used to determine the concentrations of each of the species in the solution, based on appropriate calibration curves. At plasma GSH concentration (2 μ M), both G3.5-CO-GS-S-NAC and G4-NH-CO-Pr-S-S-NAC conjugates were very stable and they did not release any detectable levels of free NAC within 17 hours period. For both conjugates 1% of NAC payload was found in the release medium in reduced GS-S-NAC or NAC-NAC forms (data not shown). The limited release of NAC in reduced GS-S-NAC or NAC-NAC forms was very rapid and completed within 1 hour. The remaining NAC stayed intact throughout the release study due to depletion of reduced GSH in the media. This suggests that NAC releases from the conjugate rapidly but the amount of NAC released will be governed by the amount of reduced GSH available.

Expected release mechanisms of both PAMAM-NH-CO-Pr-S-S-NAC and PAMAM-CO-GS-S-NAC conjugates should be similar, the only difference being the linker used. In the presence of excess GSH, the conjugates containing disulfide bonds can get shuffled by GSH in two possible ways. The conjugates may release NAC in free form while a GSH will attach onto the dendrimers forming the disulfide bond. The other possible way includes releasing of GS-S-NAC while the dendrimers will have a free thiol group. The GS-S-NAC formed can be further shuffled by excess GSH present and can yield GSSG and NAC. The shuffling reactions will reach equilibrium where the concentration of each species is will be stabilized. These fast shuffling reactions will not change the total number of disulfide linkages while slow oxidation reactions can also take place forming new disulfide bonds.

Both conjugates were analyzed for their release characteristics at intracellular GSH concentration (10 mM). The results suggest that both conjugates were able to release significant amounts of free NAC within an hour (Figure-6). PAMAM-NH-CO-Pr-S-S-NAC conjugate released 47% of NAC payload in free form within 1 hour. Additionally 19% of NAC payload was found in GS-S-NAC oxidized form. The total NAC that was detached from the dendrimers within 1 hour was 66%. The extent of NAC released did not change significantly after the initial release within 1 hour; 41% of NAC payload was found in free form and 14% was found as GS-S-NAC. The slight decrease in NAC content was most likely due to the error in concentration determination and it was within standard error limits. Similarly, PAMAM-CO-GS-S-NAC conjugate released 39% of NAC in free form and another 6% in GS-S-NAC form within 1 hour, yielding a 45% total NAC release. At 17 hours the free NAC content was determined as 46% and 6% of NAC payload was in GS-S-NAC form. At intracellular GSH concentration, no NAC-NAC was formed throughout the release studies. Absence of NAC-NAC can be explained by the excess amount of GSH present in the media, which can transfer the disulfide linkage onto either GSSG or GS-S-NAC by disulfide exchange reactions. After the initial rapid release of NAC the concentrations of analytes did not change significantly, which suggests that the cleavage is by fast exchange reactions which reach equilibrium within 1 hour. The difference in drug release of the two conjugates may be explained by the different types of linkers used for attachment of NAC.

Our study shows fast release of NAC from the conjugates in intracellular GSH levels and the stability at plasma GSH levels, these results suggest the similar release mechanism for NAC from the conjugates. This study demonstrates that PAMAM dendrimers based NAC delivery

systems can be developed for various applications. The above results have significant implications in both designing dendrimer-based drug delivery systems. Enzymatic release of drugs from dendritic delivery systems is challenging. Smaller generations were shown to be capable of enzymatic cleavage but lower generations lack the enhancements higher generations have to offer, whereas higher generations face steric hindrance issues (43,44). Commonly used pH responsive release systems usually provide slower drug release over longer time periods unless the release takes place at a very low pH (24,44). The two GSH responsive delivery systems described here have very fast release kinetics at intracellular conditions and demonstrate that GSH can be used as a reliable releasing agent in dendrimers-based delivery systems. Interestingly, the thiol containing drugs are capable of forming the disulfide bonds and this is one major contributing factor for their enhanced protein binding and reduced bioavailability. Further this study shows that the covalent linking of the thiol containing drugs by disulfide bonds would provide a means of releasing these drugs from the carrier systems at the targeted sites.

Cell Entry of Conjugates in BV-2 Microglial Cell

Cellular entry of FITC-PAMAM-NH-CO-Pr-S-S-NAC conjugate was evaluated using flow cytometry and confocal microscopy. The flow cytometry shows a significant fluorescence intensity increase within 15 min, as evidenced by two-orders of magnitude increase in the fluorescence intensity (Figure 7). Subsequently, there was a moderate increase in fluorescence noted till 120 minutes. This shows that the FITC-PAMAM-NH-CO-Pr-S-S-NAC conjugates entered the cells rapidly.

The cellular entry was also visualized using confocal microscopy (Figure 8). It is evident that the FITC-PAMAM-NH-CO-Pr-S-S-NAC conjugate enters the cells and localizes mostly in the cytoplasm, while the nucleus appears to be relatively free of the presence of any fluorescence at this time scale. The cells without any treatment show no fluorescence. From the above results, it appears that FITC-PAMAM-NH-CO-Pr-S-S-NAC conjugates are transported inside the cells efficiently, perhaps by endocytosis (45).

Efficacy assay of conjugates

NAC exerts its therapeutic effects by decreasing the production of pro-inflammatory cytokines, and reactive oxygen and nitrogen species. In our *in vivo* studies, we seek to use NAC to treat neuroinflammation induced by activated microglial cells in perinatal brain injury. Therefore, we evaluated the cellular efficacy of these conjugates in the BV-2 mouse microglial cell line that is activated by LPS. Microglial cells activated by LPS release the free radical NO that can result in damage to membranes and DNA of the surrounding cells leading to cell death. The anti-oxidative properties of the conjugates were tested by measuring the nitrite levels as a marker of free radical NO production in the cell supernatant. Free NAC inhibited nitrite production in a dose-dependent manner after 72 hours of incubation. At 24 hours, a time point at which a relatively lower amount of NO is produced, only the highest concentration of free NAC (8 mM) inhibited nitrite release. Both PAMAM-NH-CO-Pr-S-S-NAC and PAMAM-CO-GS-S-NAC conjugates showed significant inhibition of nitrite production even at the lowest equivalent dose of NAC (0.5mM). In fact, in both conjugates, with anionic and cationic terminal groups, 0.5mM NAC in the conjugated form showed equivalent efficacy to 8mM of free NAC. The conjugates did not show a significant dose dependence, at the three concentrations equivalent to free NAC, perhaps because significant suppression (>60%–80%) was seen even at the lowest concentrations (Figure-9) for both conjugates. Perhaps, lower concentration of conjugates may enable us to find dose dependence, but detailed dose-dependence is beyond the scope of this study. At equivalent concentrations, the cationic PAMAM-NH₂-NAC conjugate showed slightly better efficacy than the anionic PAMAM-COOH-NAC conjugate. From these results, it appears that improved intracellular uptake and

high drug payload in the dendrimer conjugate may be producing a high local drug concentration inside the cell to elicit a significant therapeutic response. It also suggests that an appreciable amount of the drug is released intracellularly even at these relatively short time intervals (especially for polymer conjugates).

The above results have significant implications in both understanding and manipulating drug release mechanisms, and achieving controlled intracellular drug release in dendrimer-based delivery systems. The results demonstrate that GSH can be used as a reliable *in vivo* releasing agent in dendrimer-based delivery systems. As the most abundant thiol species in living cells, GSH is the most likely candidate for the disulfide reduction in previously reported drug delivery systems. The relatively rapid drug release at intracellular GSH levels is key in dendrimer-based conjugates where drug release is typically much slower. The manipulation of GSH concentration in living cells as demonstrated here conclusively proves that GSH-mediated release is a viable mechanism for releasing payloads from dendrimer conjugates.

Conclusions

We have developed a dendrimer-NAC conjugate for the treatment of neuroinflammation associated with cerebral palsy in perinatal applications. The PAMAM dendrimer-based intracellular drug delivery system uses a linker that uses GSH as the releasing agent. Two conjugates were prepared, one based on an anionic PAMAM G3.5-COOH dendrimer and one based on a cationic PAMAM-G4-NH₂ dendrimer. NMR, MALDI, and HPLC showed that the conjugate synthesis was effective and successful. *In vitro* release studies at different GSH levels, have shown that GSH is responsible for releasing payloads from dendrimer carrier in buffer. Flow cytometry and confocal microscopy revealed that the conjugates enter the cells rapidly and localize in the cytoplasm. The efficacy was assessed in activated microglial cells using nitrite inhibition. Both conjugates showed significant efficacy even at drug levels 16 times lower than that of free drug. These studies address a key challenge that relates to drug release from polymer in general, and dendrimers in particular. We have recently reported the intrinsic ability of PAMAM dendrimers to target activated microglial cells in animal models of neuroinflammation (44). Combined with the findings of these studies, which allow us to tailor the intracellular release based on glutathione levels, we will be able to design dendrimer-drug conjugates with increased *in vivo* efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MALDI-TOF analysis of modified PAMAM dendrimers to determine the average number of coupled Pr-S-S-NAC groups. PAMAM-NH₂ before (**A**) and after (**B**) reaction with SPDP and followed by NAC reaction

Figure 2.
RP-HPLC analysis of the derivatization of PAMAM –NH₂ dendrimer with the heterobifunctional cross-linker SPDP and followed by NAC reaction. PAMAM-NH₂ (**A**)
PAMAM-NH-CO-Pr-S-S-NAC (**B**)

Figure 3. MALDI-TOF analysis of modified PAMAM-COOH dendrimers to determine the average number of coupled GS-S-NAC groups. PAMAM-COOH (A) and PAMAM-CO-GS-S-NAC (B)

Figure 4.
RP-HPLC analysis of the derivatization of PAMAM dendrimer with the GS-S-NAC.
PAMAM-COOH (**A**) PAMAM-CO-GS-S-NAC (**B**)

Figure 5. RP-HPLC UV absorbance Chromatograms at 210nm (Arbitrary AU units) a. NAC b. NAC and NAC-NAC c. GSH d. GSH and GSSG e. GSH, GSSG, NAC and GS-S-NAC.

Figure 6.
Release of NAC and GS-S-NAC from conjugates (in PBS with 10mM GSH).

Figure 7.

Flow cytometry of the cell entry dynamics of FITC-PAMAM-NH-CO-Pr-S-S-NAC- conjugate in BV-2 microglial cell line. The log of FITC absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The rapid increase in the cellular uptake of FITC-PAMAM-NH-CO-Pr-S-S-NAC within 15 min is evident.

Figure 8. Confocal microscopy images (630×) after 2 hours of treatment with (Figure-8A) control, (Figure-8B) FITC-PAMAM-NH-CO-Pr-S-S-NAC. The FITC-PAMAM-NH-CO-Pr-S-S-NAC conjugates appear to be mainly localized in the cytoplasm while the nucleus appears to be relatively free of the presence of any fluorescence at this time scale.

Figure 9.

Efficiency assay of dendrimer-NAC conjugates. BV-2 cells were treated with 100 ng/mL of LPS and the indicated concentration of NAC, PAMAM-NH-CO-Pr-S-S-NAC (A) conjugate and PAMAM-CO-GS-S-NAC (B) conjugate for 3 hours. Then, incubated with 100 ng/mL of LPS for 24 and 72 hours. Nitrite in culture medium was measured using Griess reagent system. Data are mean $\pm$ SD of three samples per group, and assessed by *t* test.

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Table-1
Molecular weight estimation (by ¹H-NMR, MALDI-TOF, and ESI-MS) of NAC, and PAMAM-NAC conjugates

	Generat ion number	Molecular weight by (NMR / MALDI-TOF / ESI-MS)	Pay load	Purity of conjugate	HPLC Elution time	Solubility in PBS/H ₂ O
G4-NH ₂	4	14.1 kDa	-	100%	14.2	Highly Soluble
Pr-NAC	-	250 Da	-	-	-	-
G4-NH-CO-Pr-S-S-NAC	4	18.3 kDa	16	99.2%	15.0	Highly Soluble
FTTC	-	389 Da	--	99.5%	-	Not Soluble
FTTC-G4-NH-CO-Pr-S-S-NAC	4	19.0 kDa	18	99.5%	16.0	Highly Soluble
G3.5 -COOH	3.5	11.1 kDa	-	100%	8.25	Highly Soluble
GS-S-NAC	-	468 kDa	-	99.1%	-	Soluble
G3.5-CO-GS-S-NAC	3.5	19.7 kDa	18	99.5%	12.5	Highly Soluble