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# Effects of branching architecture and linker on the activity of hyperbranched polymer-drug conjugates

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## Abstract

Drug release from hyperbranched polymer-drug conjugates and the subsequent activity are influenced by the branching architecture and the linker. To gain an understanding of these effects, we used hyperbranched polyol and G4-OH Polyamidoamine (PAMAM) dendrimer with methyl prednisolone (MP) as the model drug. The drug was conjugated to dendrimer or polyol using a glutaric acid (GA), or a succinic acid (SA) spacer. Drug payload was the highest with polyol, while in the case of dendrimer, a higher payload was achieved with the GA than the SA spacer. Cell uptake of the polymer conjugates in A549 lung epithelial cells was higher than the free drug and the conjugates largely localized in the cytosol. The anti-inflammatory activity of polymer conjugated-MP, as measured by inhibition of prostaglandin synthesis, was the highest for MP-SA-dendrimer conjugate followed by MP-GA-polyol conjugate, and then MP-GA-dendrimer conjugate. This study suggests that the branching architecture and spacer influence the drug payload and pharmacological activity of a drug-nanopolymer conjugate, which may significantly influence the in vivo efficacy of these nanodevices. This has key implications in the eventual *in vivo* efficacy of these nanodevices.

**Synopsis—**The influence of branching architecture and spacer on drug loading and pharmacological activity of drug-polymer conjugates is reported using methyl prednisolone as a model drug. The drug loading and anti-inflammatory activity was higher with imperfectly branched polyol in comparison to perfectly branched G4OH dendrimer. Similarly the drug loading and anti-inflammatory activity was higher with succinic acid spacer compared to glutaric acid spacer in dendrimer conjugates.

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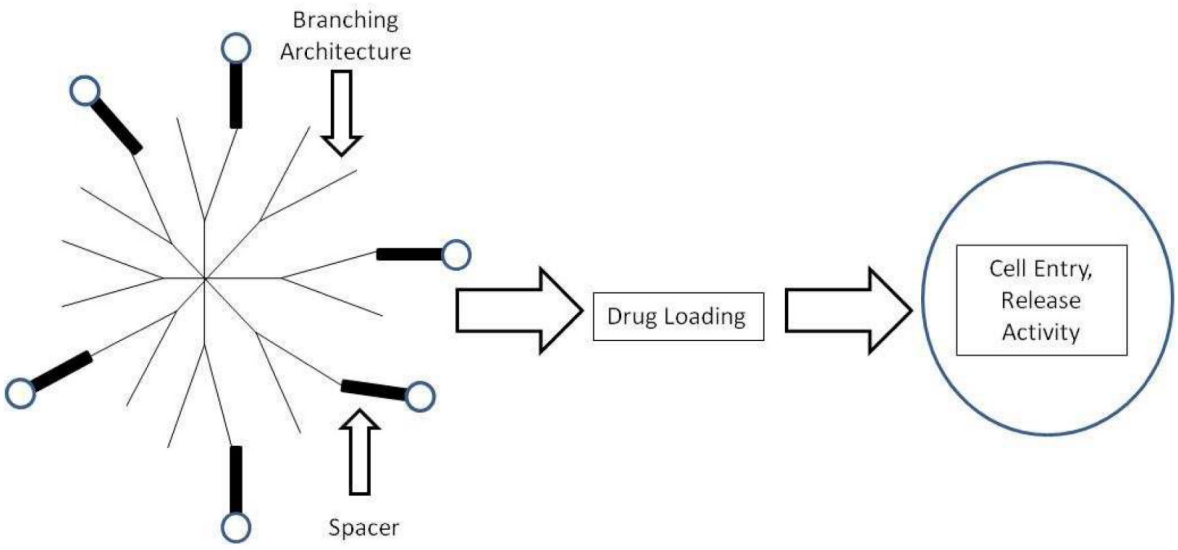


Polymeric-drug conjugates are used to modulate drug pharmacokinetics and target the drug to the desired site (1,2). Various linear polymer-drug conjugates have been investigated for improving the circulation half-life and intracellular targeting of drugs (2). However, linear polymers have limited drug payload capacity. Hyperbranched polymers (HBPs) can carry multiple copies of drug and/or targeting/imaging ligands (3). Depending on their branching architecture, these polymers are classified as 'imperfectly branched' polymers or HBPs and 'perfectly branched' polymers or dendrimers (4). Dendrimers are well-defined and monodisperse , with a controlled branching architecture. In comparison to dendrimers, HBPs are polydisperse, but their ease of synthesis (typically using one-pot reaction) makes them relatively cheaper to produce than dendrimers (5,6). Both these polymers have core-shell architecture and can carry high drug payload due to the large number of tunable surface functional groups (7,8). Earlier studies have shown that both HBPs and dendrimers can carry a high drug payload (7,8). High drug payload polymeric conjugates were prepared with small molecules such as ibuprofen by directly conjugating the drug to the surface functional groups in HBPs and dendrimers (7,8). However, the high density of surface functional groups poses steric hindrance, thus limiting the payload for large drug molecules such as steroids (9). Chemical spacers can be used to link the drug to the polymer to overcome the steric hindrance to a certain extent (10). Further, the spacers can also modulate the drug release from the polymer (10). In the present study, we investigated the conjugation of a model steroid drug, methylprednisolone (MP) to polyol and hydroxyl terminated polyamidoamine dendrimer (G4-OH) using glutaric acid (GA) and succinic acid (SA) as spacers. Comparative studies between linear and branched polymers have been reported in the literature (11).To our knowledge, this is the first report that compares the drug payload and pharmacological activity of drug-polymer conjugates between HBPs and dendrimers. The influence of the branching architecture of these two polymers and spacer on cell uptake and anti-inflammatory activity was tested in A549 human lung epithelial carcinoma cells.

Glutaric acid **2** (GA) was conjugated to $C_{21}$ hydroxyl group of MP **1** using dicyclohexylcarbodiimide (DCC) as the coupling agent (Figure 1). This hydroxyl group is more favorable compared to $C_9$ (secondary) and $C_{11}$ (tertiary) hydroxyl groups. The by-product dicyclohexyl urea (DCU) was removed by filtration. In the next step MP-GA conjugate **3** was dissolved in anhydrous dimethyl sulfoxide (DMSO) and conjugated with polyol **4** or G4-OH dendrimer in presence of DCC to form polyol-GA-MP conjugate **5** or dendrimer-GA-MP conjugate (9). The solution was filtered to remove DCU and dialyzed against DMSO to remove unreacted components. The conjugate was characterized by $^1$H NMR. A similar procedure was

followed using succinic acid (SA) spacer where methyl prednisolone succinate (MPS) was conjugated to G4OH dendrimer. For cell uptake studies both the free drug and the drug-polymer conjugate was further conjugated with fluoroisothiocyante (FITC) using DCC as the coupling agent (9). The absence of free FITC was confirmed using TLC (12). Because of the use of homobifunctional spacers, there could be a possibility of intermolecular crosslinking between dendrimers, leading to dimers and larger particles. However, since we use dilute concentrations, this is not observed, as evidenced by HPLC and MALDI of similar conjugates using glutaric acid spacers, reported previously (9).

Figure 2 shows the $^{1}$H NMR spectrum of MP-GA-polyol conjugate. Broad singlet at δ 4.2 ppm corresponds to $CH_2$ protons of polyol, while the multiplets between δ 5.5 and 7.3 correspond to protons of MP. The NMR spectra for the other conjugates can be found in the supporting information. On an average 25 molecules and 12 molecules of MP were conjugated to polyol and G4-OH dendrimer respectively using GA as the spacer (Table 1). On the other hand, 6 molecules of MP were conjugated to G4-OH using SA as the spacer. The polyol used in the present study has more functional groups than dendrimer (128 vs 64 groups) and this resulted in a higher drug loading in polyol. Furthermore, the 'imperfect branching' architecture in polyol reduced the steric hindrance for conjugation of MP-GA to polyol. SA has a shorter alkyl chain than GA, which can also contribute to the difference in drug payload between the two dendrimer conjugates. Further MPS was used as the sodium salt and therefore the difference in acidity may also contribute to the difference in reactivity and lower drug payload with SA spacer. Nevertheless, the drug payload using MPS was higher than when free MP was directly conjugated to dendrimer (9). On an average 4-5 molecules of FITC were attached to the conjugates.

The cell uptake of FITC-labeled free drug and the conjugates was determined by measuring the intracellular fluorescence intensity using flow cytometry. Equivalent MP concentrations were used for uptake studies. With respect to zero time point, the fluorescence intensity was found to increase with time for free MP and MP-polymer conjugates (Figure 3). However, the cell uptake of the conjugates appears to be significantly higher ($p<0.05$) than the free MP. This is despite the fact that the FITC concentration is higher for free MP compared to the conjugates (Each MP molecule had a FITC attached to it, whereas the conjugates have approximately half the FITC for equivalent MP concentration). The uptake profiles for the two glutaric acid spacer conjugates (MPGD and MPGP) are not appreciably different (Figure 3). However, the conjugate with succinic acid spacer (MPSG) showed higher cell uptake than the other two conjugates. Both free MP and polymer conjugated MP was predominantly localized in the cytosol (Figure 4a-e). In case of the conjugates, there was extensive fluorescence around the perinuclear region, indicative of endocytotic uptake of the conjugates as opposed to diffuse fluorescence distribution with the free drug. Free MP enters the cell by passive diffusion and cell entry by passive diffusion is non-specific, which is dictated by the concentration gradient between the extracellular and intracellular milieu (13). Unlike the free drug, the polymer conjugates enter the cell by specific endocytosis processes. The rate of endocytosis of polymers is influenced by a number of factors including molecular weight, surface charge and hydrophilicity of the polymer (13,14). We have previously shown that at the concentrations used in this study, the polymers were found to be non-toxic to A549 cells (15). The difference in the hydrophilicity of SA and GA can be attributed to the difference in uptake of the dendrimer conjugates. On the other hand, the branching architecture did not appear to influence the cellular uptake of the conjugates, as the polyol and the dendrimer conjugates show a comparable uptake profile. This is not unexpected, as dendrimer and polyol do not differ much in their molecular weight and surface charge (Table 1). Both G4-OH dendrimer and polyol have no net surface charge and hence are expected to be transported by fluid phase endocytosis through non-specific interactions with the cell membrane (15).

The anti-inflammatory activity of conjugates was evaluated by their ability to inhibit cyclooxygenase enzyme (Cox-2) and suppress prostaglandin ($PGE_2$) synthesis in A549 cells. There was no significant difference in the activity of free and polymer conjugated drug at 5μg/ml equivalent concentration of MP (Figure 5a). This is a significant result, since most polymer-drug conjugates show significantly less activity than free drugs in cells, even though they show better efficacy *in vivo*. The $PGE_2$ inhibition observed in the present study is consistent with the maximal inhibition reported for MP in A549 cells (16). When the activity was tested at a concentration (5ng/ml) close to the $IC_{50}$ of MP in A549 cells (16), the conjugates showed lower activity than free MP. Particularly, MP-GA-dendrimer conjugate showed significantly lower activity than the other two conjugates. Since an equivalent concentration of MP was used, the results show that the spacer has an influence on the release of free MP from the polymer. To further understand the influence of branching architecture, the activity was measured at shorter treatment time of 1 hour. At short times, both the dendrimer and the polyol conjugates show lower activity than the free drug (Figure 5b). At later time points, the diffusion produces higher intracellular concentration, whereas the drug release from the conjugates is a much slower process. This may result in efficacies being comparable at the later time point.

As shown in Figure 5b, at shorter treatment time, there was a significant difference in the activity of polyol and dendrimer conjugates. The higher activity of polyol conjugates suggests that the imperfect branching architecture may allow easy access of the lysosomal enzymes to release the drug faster compared to the perfect branching architecture in dendrimer. At the same time, the polydispersity of polyol may also contribute to the difference in the activity of the conjugates.

Lysosomes in the cell are rich in enzymes that aid in releasing the free drug from the polymer conjugate (17). The release of free drug from the conjugate is influenced by many factors including the type of spacer, the stability of the chemical bond, distance of the spacer from the polymer backbone, the hydrophilicity of the surrounding medium and steric overcrowding at the center of reaction (18). Both GA and SA have two ester bonds that connect the drug and polymer and can be cleaved by esterases. However, GA differs from SA by one carbon atom. This difference in the alkyl chain length seems to influence the enzymatic release of the free drug. Previously, Wiwattanapatapee et al (19) have shown that the release of 5-amino salicylic acid from dendrimer conjugate is influenced by the carbon chain length in the spacer. Tang et al (20) found that the DNA expression was enhanced when the dendrimer was scrambled by heat treatment. The authors attributed this effect to the increased conformational flexibility of dendrimer, which enabled DNA complexation and its subsequent release from the complex. Recently, Khandare et al (11) reported that the release of paclitaxel in presence of esterase was faster from linear polyethylene glycol (PEG) conjugate compared to dendrimer conjugate. On the other hand, the $IC_{50}$ for anti-proliferative activity of paclitaxel-dendrimer conjugate was 300 times lower than the paclitaxel-PEG conjugate. Since polyol has a higher drug payload, with the same given dose of the conjugate, polyol-MP conjugate will provide higher intracellular concentration when compared to the dendrimer-MP conjugate. Similarly, the drug loading and release of drug from the dendrimer-drug conjugate can be modulated by suitable choice of spacer. Further studies into the release kinetics of MP from the conjugates will provide better insights. However, the preliminary findings presented here show that drug payload and pharmacological activity of drug-polymer conjugates is influenced by the branching architecture and spacer chemistry and these factors need to be considered in designing specific nanodevices for various therapies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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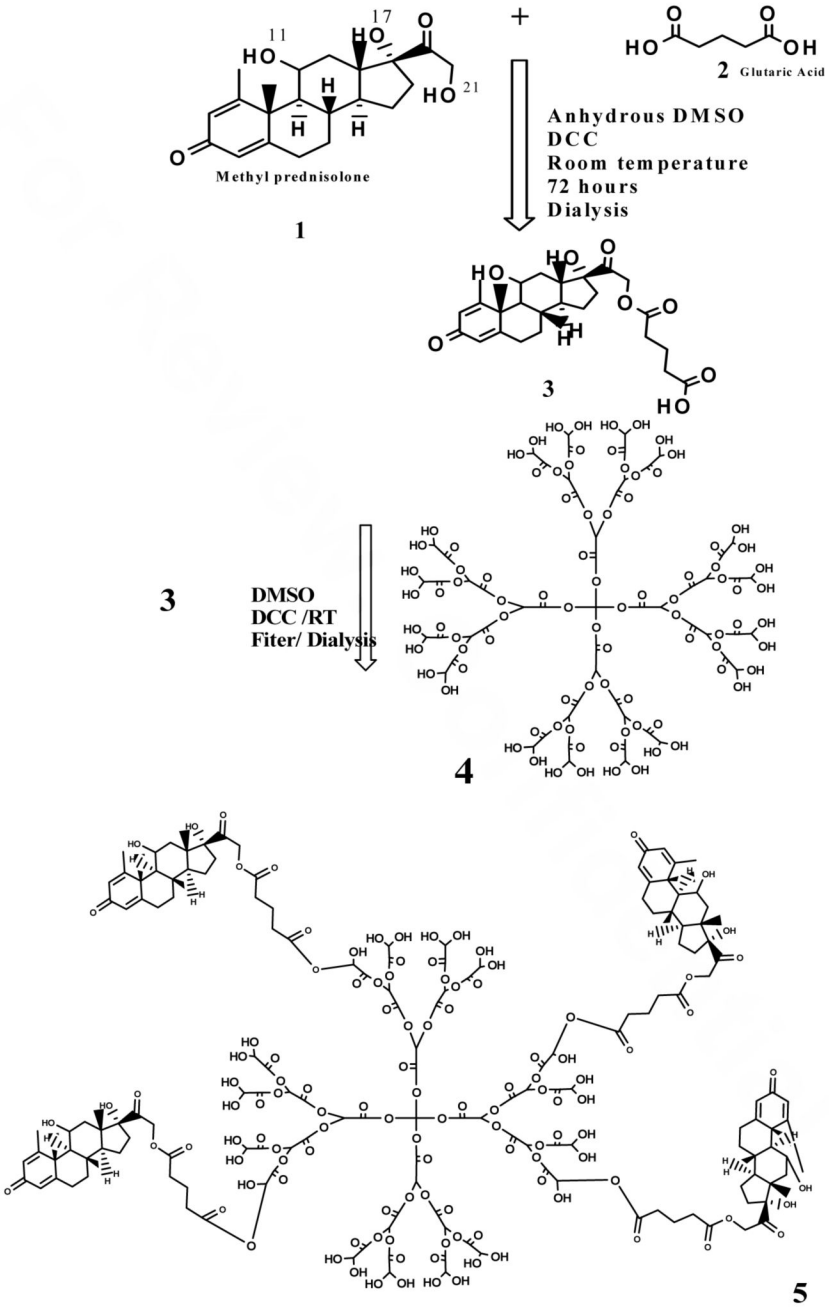


**Figure 1.**
Synthetic scheme for methylprednisolone-glutaric acid-polyol conjugate.

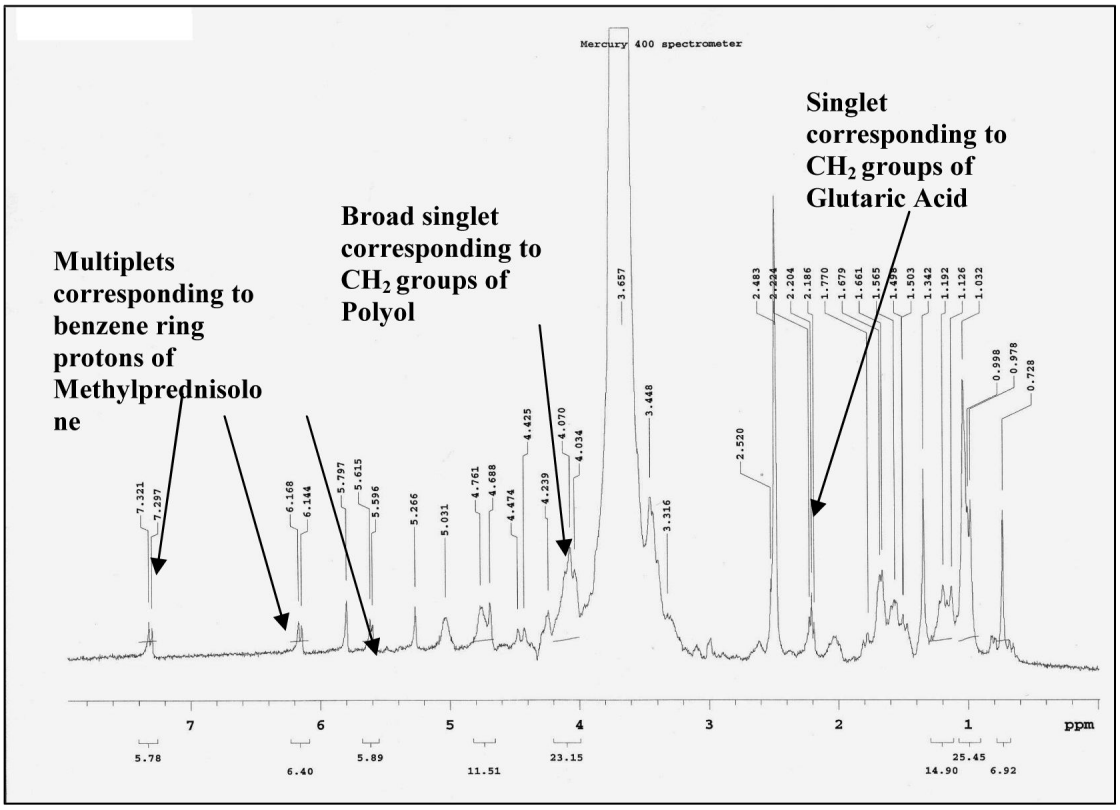


**Figure 2.**

$^1$H NMR spectrum of methylprednisolone-glutaric acid-polyol conjugate. The corresponding proton peaks are indicated in the spectrum. The spectra for the other conjugates are analyzed in a similar manner, and are included for the sake of brevity. Proton integration method is used to calculate the drug payload.

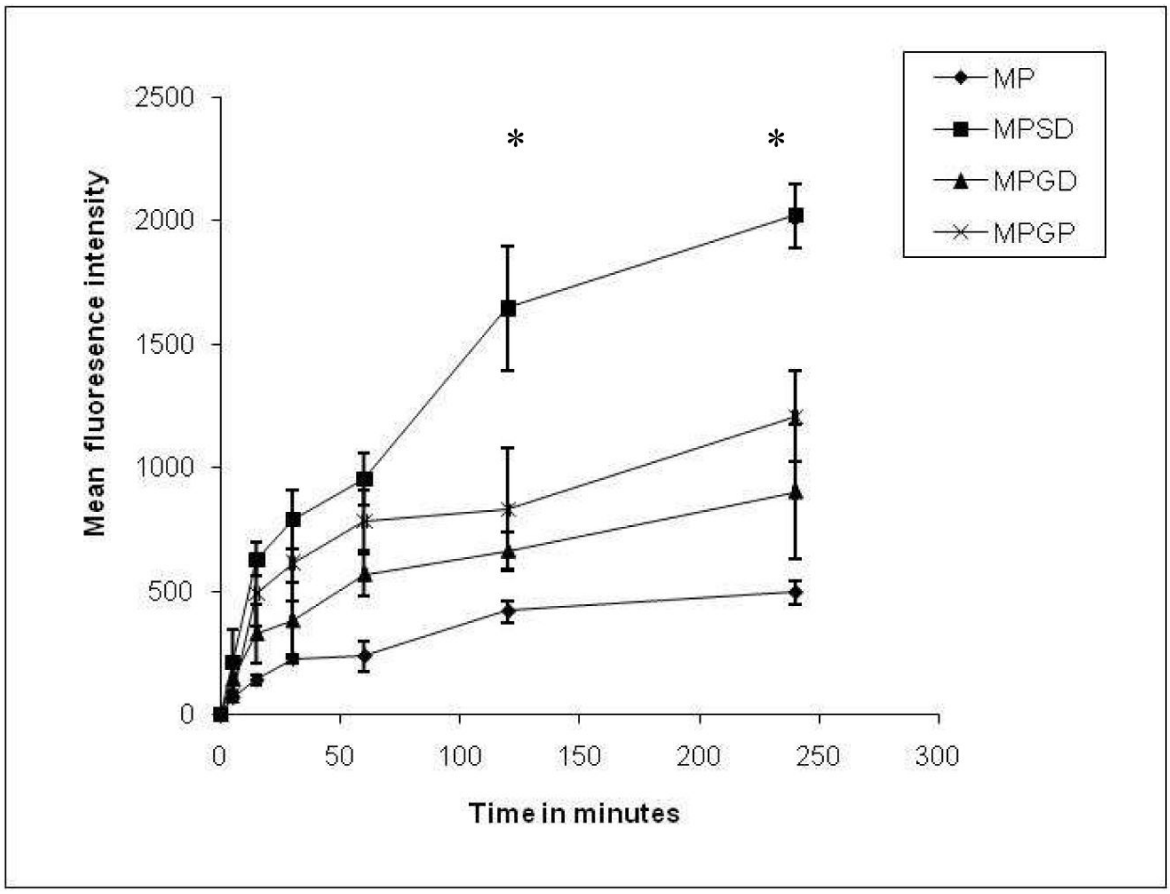


**Figure 3.**
Cell uptake of free MP and polymer conjugated MP in A549 cells. The y-axis indicates the mean fluorescence intensity as measured by flow cytometry, subtracting the autofluorescence at zero time. Each data point represents average of three measurements with standard error bars. Key: MP-methylprednisolone; MPSD-methylprednisolone-succinic acid-dendrimer, MPGD-methylprednisolone-glutaric acid-dendrimer, MPGP-methylprednisolone-glutaric acid-polyol, *Statistically significant ($p<0.05$) for MPSD.

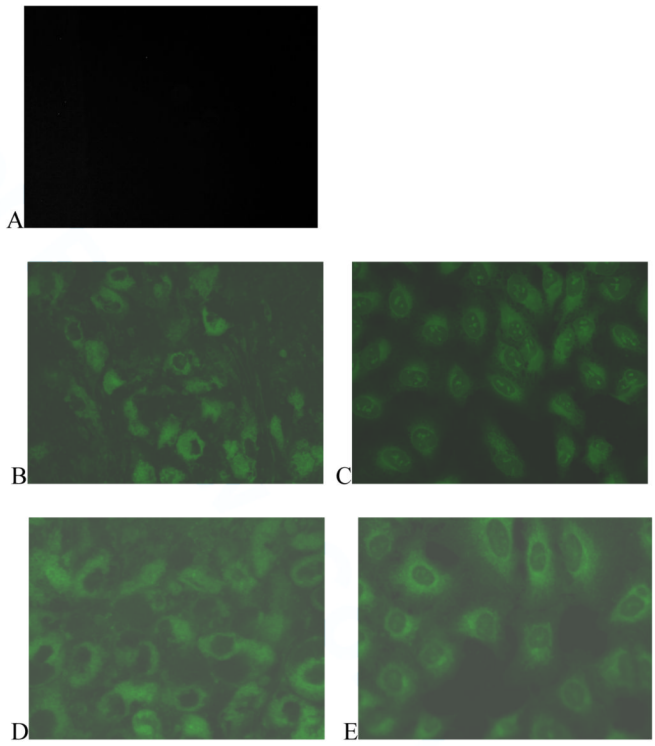


**Figure 4.**
Fluorescent microscopic images (63 × 1.2) of A549 lung epithelial cells after 1 hour of treatment with A) control cells with no treatment; B) MP-FITC, C) MP-succinic acid-dendrimer-FITC, D) MP-glutaric acid-dendrimer-FITC, E) MP-glutaric acid-polyol-FITC.

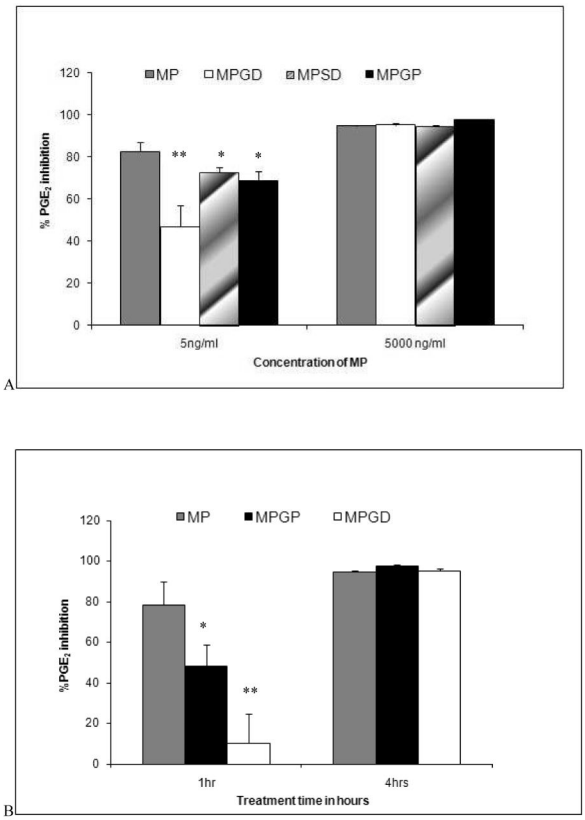


**Figure 5.**

Inhibition of prostaglandin ($PGE_2$) synthesis after treatment with free drug and drug-polymer conjugates in A549 cells. A. Percent inhibition of $PGE_2$ synthesis after treatment with two different concentrations of methyl prednisolone. The cells were treated with free drug or drug-polymer conjugates for 4 hours. B. Percent inhibition of $PGE_2$ synthesis after treating the cells for two different time periods with 5μg/ml equivalent of methyl prednisolone. The percent inhibition represents the reduction in $PGE_2$ levels in comparison to control (A549 cells with prostaglandin induction but no treatment). Key: MP-free methyl prednisolone; MPSD-methylprednisolone-succinic acid-dendrimer; MPGD-methylprednisolone-glutaric acid-dendrimer; MPGP-methylprednisolone-glutaric acid-polyol; *statistically significant ($p<0.05$) when compared to the free MP treatment, ** statistically significant when compared to other conjugates. All the values are mean of four measurements with standard error bars.

## Table 1

Comparison of molecular weight and number of molecules of drug conjugated to polymers

| Polymer | Molecular weight (Da) | No. of end groups | *Percent Weight of MP in conjugate | *Av. no. of MP per molecule of polymer |
|---|---|---|---|---|
| G4-OH dendrimer | 14,279 | 64 | - | - |
| Polyol | 15,000[a] | 128 | - | - |
| MP-GA- dendrimer | 21,395 | - | 32 | 12 |
| MP -SA-PAMAM dendrimer | 16,790 | - | 17 | 06 |
| MP-GA-polyol | 27,550 | - | 45 | 25 |

MP- methylprednisolone, SA-succinic acid, GA- glutaric acid

[a]From Perstorp Polyol literature

*Estimated from $^{1}$H NMR. The molecular weights reported here are the approximations based on the number of molecules of MP conjugated to the polymer