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ORIGINAL RESEARCH

Tumor cell imaging using the intrinsic emission from PAMAM dendrimer: a case study with HeLa cells

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Abstract HeLa 229 cells were treated with methotrexate (MTX) and doxorubicin (DOX), utilizing fourth generation (G4), amine terminated poly(amidoamine) {PAMAM} dendrimer as the drug carrier. In vitro kinetic studies of the release of both MTX and DOX in presence and absence of G4, amine terminated PAMAM dendrimers suggest that controlled drug release can be achieved in presence of the dendrimers. The cytotoxicity studies indicated improved cell death by dendrimer-drug combination, compared to the control experiments with dendrimer or drug alone at identical experimental conditions. Furthermore, HeLa 229 cells were imaged for the first time utilizing the intrinsic emission from the PAMAM dendrimers and drugs, without incorporating any conventional fluorophores. Experimental results collectively suggest that the decreased rate of drug efflux in presence of relatively large sized PAMAM dendrimers generates high local concentration of the dendrimer-drug combination inside the cell, which renders an easy way to image cell lines utilizing the intrinsic emission properties of PAMAM dendrimer and encapsulated drug molecule.

Keywords PAMAM dendrimer · Methotrexate · Doxorubicin · HeLa 229 cells · Drug release

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Introduction

The emergence of employing dendritic and hyper branched polymeric structures as drug and gene carriers into cells has resulted in a plethora of investigations in biomedical fields during the past two decades (Majoros et al. 2005). The unique features of dendrimers compared to the traditional polymers have emanated from their defect-free, three dimensional molecular architecture, which satisfy the necessary requirements for efficient drug-encapsulation and controlled drug-release in both in vitro and in vivo systems (Esfand and Tomalia 2001). Poly(amidoamine) {PAMAM}dendrimers with nearly spherical structures have been widely studied for drug delivery purpose, both in vitro and in vivo, and various generations of PAMAM dendrimers with different core and peripheral groups are commercially available (Najlah et al. 2007).

The high density of the peripheral groups in relatively higher generation PAMAM dendrimers is

expected to have potential applications in enhancing the solubility of numerous drugs in aqueous media. Due to the large size, encapsulation of drug molecules is possible either in the interior part or on the periphery of PAMAM dendrimer (Emanuele and Attwood 2005). Since 1995, many research groups have been proposing the possibility of constructing a dendritic 'core–shell molecule' for entrapping small molecules (Jansen et al. 1995). Theoretical calculations as well as computational studies on PAMAM dendrimers showed that for higher generations, these dendrimers adopt a globular structure with large hollow cavities inside, where drug molecules can be encaspualted (Chai et al. 2001, 2005; Maiti et al. 2005; Maiti and Goddard 2006). An ideal dendrimer based drug delivery system must be non-toxic, non-immunogenic and biodegradable. The use of PAMAM dendrimers as modulators of the release of incorporated drugs and the possible alterations of the drug bioavailability has attracted considerable attention in biomedical research field.

Quite a few attempts have been reported to enhance the efficiency of drug loading and delivery by PAMAM dendrimers for treating cancer cells (Choi et al. 2005; Gurdag et al. 2006; Majoros et al. 2003). Functionalized dendrimers were connected via DNA oligonucleotides to generate clustered molecules targeting cancer cells (Choi et al. 2005). Majoros et al. have recently reported that partial acetylation of fifth generation PAMAM dendrimer leads to enhanced solubility during the conjugation reaction between fluorescein isothiocyanate and the dendrimer, which prevent non-specific targeting of cancer cells during drug delivery (Majoros et al. 2003). Several attempts have been made to encapsulate anti cancer drugs such as methotrexate and doxorubicin inside PAMAM dendrimers and the results have shown that up to twenty-six drug molecules can be incorporated per dendrimer monomer (Emanuele and Attwood 2005; Gurdag et al. 2006). PAMAM dendrimers have been widely used as a carrier for various drugs for the treatment of lymphoblastoid leukemia, Chinese hamster ovary cell lines, and KB cells (Thomas et al. 2005).

The present work initiates the study of controlled drug delivery to the HeLa 229 cell line, utilizing fourth generation PAMAM dendrimer as the drug carrier and methotrexate (MTX) and doxorubicine (DOX) as the two anti-cancer drugs. The PAMAM-drug combination showed better in vitro cytotoxic activity and a delayed release of drugs compared to control experiments with drug alone, which is essential for reducing the side effects and increasing therapeutic index. More importantly, the intrinsic emission property of PAMAM dendrimer and drugs molecules is effectively utilized for imaging the cell line through fluorescence microscopy.

Materials and methods

Materials

Methanol solution of starburst amine terminated fourth generation PAMAM dendrimer, MTX, DOX, Verapamil and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich company. Fetal bovine serum (FBS), 100× pencillin (100 U/mL), streptomycin (100 μg/mL), DMEM media and Trypsin–EDTA were purchased from GIBCO (Gaithersburg, MD). 10 KD dialysis membrane was procured from Spectrum Company. HeLa 229 cell line was obtained from National Centre for Cell Sciences, Pune, India. All other chemicals used were of analytical grade and obtained from commercial sources.

Methods

Preparation of PAMAM-MTX complex

The solvent from the commercial sample of amine terminated fourth generation PAMAM dendrimer was evaporated by purging ultra high pure nitrogen gas and then the dendrimer was dissolved in double distilled, de-ionized water. MTX solution (10^{-5} M) was added to the dendrimer solution (3.8×10^{-7} M) and stirred for 16 h at room temperature (Papagiannaros et al. 2005).

In vitro drug release study

For in vitro drug release study, 7 mL of the drug loaded dendrimer solution (3.8×10^{-7} M) was taken in a hermetically tied dialysis sac. The dialysis sac was placed in 140 mL deionized water and maintained at room temperature on a magnetic stirrer. At predetermined intervals of time, sample was

withdrawn and replaced by fresh deionized water. The UV–Vis absorption spectra of withdrawn samples were taken to determine the released drug concentration.

Cell culture

Human cervical carcinoma cell line HeLa 229 was grown continuously as a monolayer at 37 °C and 5% CO_2 in Dulbecoo's modified Eagle's medium (DMEM) supplemented with streptomycin (100 μg/mL), penicillin sulphate (100 units/mL) and 10% heat-inactivated fetal bovine serum (FBS). Cells were routinely maintained by trypsinizing the cells and plating on plastic tissue culture dishes (Corning) at 37 °C in a humidified atmosphere containing 5% CO_2.

Preparation of sample for fluorescence imaging

MTX and DOX (10^{-5} M) were loaded into amine terminated fourth generation PAMAM dendrimer in 1:1 glycerol water mixture by stirring for 16 h at room temperature. Also, solutions of methotrexate, doxorubicin and PAMAM were prepared separately in 1:1 glycerol- water mixture at same concentration for control experiment. The solutions were then filter sterilized by 0.22 μm syringe and then added to the HeLa 229 cells. HeLa 229 cells were grown in 12 well plates and then treated with the dendrimer-drug complex, the dendrimer, and the drug followed by incubating them for 24, 48 and 72 h at 37 °C in the CO_2 incubator. After the appropriate incubation time medium was removed from each well and cells were washed with Phosphate buffered saline (PBS) and fluorescence was checked in fluorescence microscope (Nikon, Eclipse Ti).

Fluorescence studies in presence of verapamil

HeLa 229 cells were grown in 12 well plates for 24 h before the treatment of drugs. After one day of cell plating cells were treated with folic acid (FA), MTX, and DOX alone and in combination with PAMAM dendrimer. To see the effect of verapamil on the fluorescence intensity of DOX, MTX and FA, these drugs were treated with the cells in presence of 100 μM verapamil. Verapamil block the efflux of these compounds and hence increases their net accumulation inside the cell. After 24 h of treatment the fluorescent intensity was checked in the fluorescence microscope.

Cell cytotoxicity

For the cytotoxicity assay, the colorimetric MTT assay was performed. Briefly, HeLa 229 cells were seeded at a density of 6×10^3 cells/well in a 96-well cell binder plate and grown in 100 μL of DMEM containing 10% FBS for 1 day prior to incubation with dendrimer-drug complex. After 1 day of incubation, the cells were treated with 0.1–10 μM concentrations of dendrimer-drug conjugate, drugs alone and dendrimer alone. The cells were incubated with the conjugates for 24, 48, and 72 h and after the incubation time MTT assay was carried out. In brief, after the 24, 48, and 72 h incubation time, medium was removed from each well and 100 μL of fresh medium were added the 10 μL of MTT stock solution (10 mg/mL) was added to each well and incubated for 4 h at 37 °C in the dark in a CO_2 incubator. After that 110 μL of stop solution (20% Sodium dodecyl sulfate in 50% Dimethyl formamide) was added, further incubated for 24 h at 37 °C and the absorbance was measured at 595 nm using a scanning multiwell ELISA reader (Microplate Autoreader EL311, Bio-Tek Instruments Inc., Winooski, VT, USA).

Statistical analysis

All experiments were conducted at least in triplicates and results are expressed as mean ± S.E. unless otherwise specified. Unpaired student's *t*-test was applied to calculate statistical significance. A difference between mean values was considered significant if $P < 0.05$.

Results and discussion

Dendrimers have been identified as suitable candidates for drug delivery purpose because: (a) they have structural flexibility, providing numerous possibilities to 'fine-tune' the functional groups at the periphery of the macromolecule and thereby modulate the dendrimer-drug interaction, and (b) they have nanometered internal cavities, where drug molecules can be encapsulated, making the drug more soluble in aqueous systems. This leads to two major approaches

for drug loading in dendritic systems; (a) attaching the drug covalently or non-covalently at the periphery of the dendrimer and (b) incorporating the drug in the interior portions of the dendrimer. The latter one is more desirable since the release of the drug molecule will be diffusion controlled whereas in the former case, suitable mechanisms have to be adopted to detach the drug from the dendrimer periphery (Ambade et al. 2005).

In the present study, in vitro release of drug molecules (MTX and DOX) from amine terminated fourth generation PAMAM dendrimer was investigated using UV–Vis absorption technique (Patri et al. 2007). The fourth generation PAMAM dendrimer was utilized for this purpose mainly because of its enhanced cell permeability nature (for instance, the permeability of different generations of dendrimers for epithelial cell monolayer follows the order G4 $>>$ G0 $\sim$ G1 $>$ G3 $>$ G2) (Florence et al. 2000). Higher than fourth generation dendrimers were not utilized because of the increased degree of back folding of the peripheral functional groups which might affect the drug loading efficiency of the system. Figure 1 provides the concentration vs time plot for the release of methotrexate from PAMAM dendrimer. The plot clearly indicates that the rate of drug release is considerably regulated, especially in the initial part of the delivery, in presence of PAMAM dendrimers. A similar trend was observed for DOX release kinetics as well (please see the supporting information).

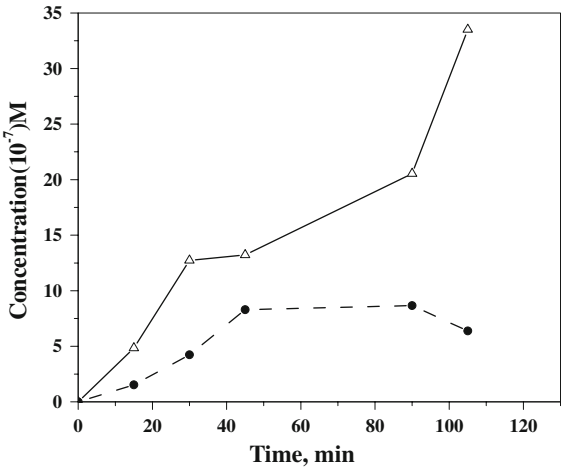

Fig. 1 Release profiles of MTX from MTX alone (*open triangle*) and from PAMAM-MTX complex (*closed circles*)

The cytotoxicity of free MTX, free DOX, PAMAM-MTX and PAMAM-DOX was determined by colorimetric MTT assay experiments. Treatment with the drugs and drug dendrimer complex was done at different time intervals and different concentrations. Figures 2 and 3 present the histogram plots of cell viability obtained from the absorbance of the cells at A595 at a concentration of the species of 10 μM.

It is clear from the experiments that the cytotoxicity of MTX-PAMAM is enhanced compared to MTX alone. PAMAM at a concentration of 10 μM has a similar toxic effect as 10 μM of MTX. The toxicity of PAMAM is originated from the large number of peripheral amino group present in the molecule. It has been reported in the literature that peripheral modification of PAMAM dendrimer by suitable moieties can substantially reduce the toxicity effect of PAMAM dendrimers (Nam et al. 2009). Nevertheless, upon complex formation with MTX, the cell death is enhanced compared to the case where the cells are treated with MTX or PAMAM alone. Similarly, the cytotoxic effect of PAMAM-DOX is significantly higher compared to DOX alone. More importantly, the relative cytotoxic effect of these drugs alone and with dendrimer complex is almost identical for both 24 and 48 h of treatment. The treatment was done at a concentration from 0.1 to 10 μM and the relative results are the same at all concentrations (please see the supporting information).

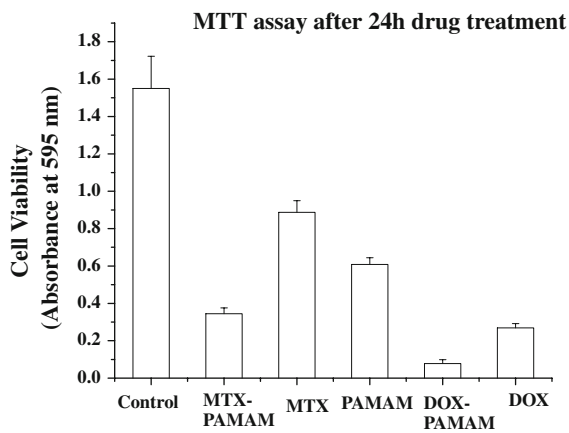

Fig. 2 MTT assay after 24 h of drug treatment. All experiments were done in triplicate and the variation in the readings were shown as *error bars*

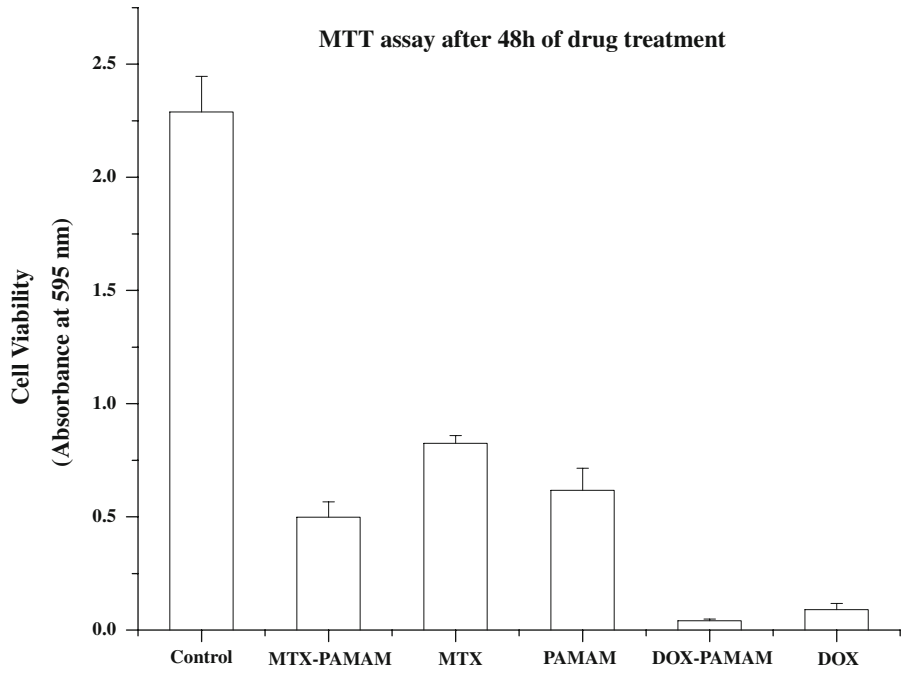

Fig. 3 MTT assay after 48 h of drug treatment. All experiments were done in triplicate and the variation in the readings were shown as *error bars*

Since imaging tumor cell lines utilizing fluorescence techniques is gaining increasing attention, it is of paramount importance to incorporate fluorescent moieties to anti-cancer drug carriers such as PAMAM dendrimers (Fahmy et al. 2007). Recent developments in the dendrimer chemistry, however, suggested that dendrimers emit intrinsic fluorescence (i.e., fluorescence in the absence of conventional fluorophores) in the bluish-green region (emission maximum = 470 nm), which can be controlled via pH, chemical oxidation and salvation (Larson and Tucker 2001). While the initial report on intrinsic emission was observed from carboxylate terminated PAMAM dendrimers, further experiments suggested that amine and hydroxy terminated PAMAM dendrimers also emit with high quantum yield (Wang and Imae 2004). These observations were followed by numerous other reports, unequivocally proving the presence of an inherent emission from amine containing dendrimers (Wang et al. 2007; Lee et al. 2004). Experimental results from our laboratory have shown that presence of glycerol, a biocompatible reagent, has increased the intrinsic emission intensity to a great extent. Thus, cell line imaging studies were carried out in glycerol-water mixtures.

It is worth noting that drug molecules such as DOX also emit intense intrinsic emission in the green region of the visible spectrum (Wartenberg et al. 1998). While the intrinsic emission from PAMAM dendrimer and drug molecules such as DOX was well recognized, attempts to utilize this for cell line imaging has not been initiated. We hypothesized that interaction between two intrinsically fluorescing molecular entities (i.e., drug and dendrimer) will result in a system with enhanced emission properties due to the overall rigidity gained by the system through complex formation. We have imaged HeLa 229 cell line, treated with PAMAM-drug complexes, utilizing the intrinsic emission from PAMAM dendrimers and drugs. Figure 4 shows the fluorescence microscopic images obtained after treating HeLa 229 cells with MTX, DOX, FA and their combination with PAMAM dendrimers. The results suggested that the intrinsic emission is enhanced upon complex formation between drugs with the dendrimers, compared to the individual cases. It is evident from the Figure that intrinsic emission can effectively be utilized to image cell lines and additional fluorophores are not necessarily attached to the dendrimers as in the conventional way. This is expected to

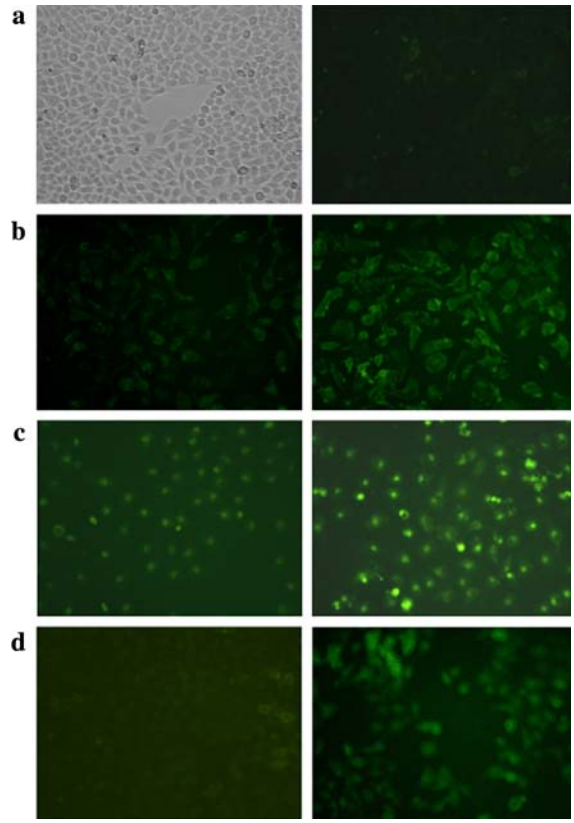

Fig. 4 Fluorescence images of HeLa 229 cell line; **a** normal view (*left*) and fluorescence view (*right*) of control cells; **b** fluorescence view of cells treated with MTX (1.0 μM) alone (*left*) and PAMAM-MTX (1.0 μM) complex (*right*), **c** fluorescence view of cells treated with DOX (10 μM) alone (*left*) and PAMAM-DOX (10 μM) complex (*right*), **d** fluorescence view of cells treated with FA (1 μM) alone (*left*) and PAMAM-FA (1 μM) complex (*right*), All the experiments were done three times. The pictures shown were the representatives of three independent experiments

tremendously minimize the difficulties associated with the synthesis and purification of dendrimer based drug carriers attached with conventional type fluorescent molecules. More importantly, this will result in the enhanced drug loading capacity for cases where dugs are attached to the periphery of dendrimers, as available free space will be more due to the absence of conventional fluorophores in the system. In addition to that, toxicity issues related to the conventional type fluorescing units can also be eliminated if intrinsic emission properties of dendrimer and drug molecules are utilized for cell imaging.

Cancer cells have the propensity to efflux the drug when given alone or when the drug molecule is small in size (Bronger et al. 2005). MDR resistance gene increases the efflux of the drug, thus decreasing its bioavailability (Raub 2006). One way to overcome this problem is to develop a system where one can reduce the efflux of the drug. An approach has been tested where the drug was encapsulated in long chain polymer and/or PAMAM dendrimers that protect the drug from efflux system (Gillies and Frechét 2005). Encapsulating the drug through this procedure allows the bypassing of the drug efflux system and increase drug solubility therefore increasing the drug bioavailability, which is required for the effective treatment. This strategy was tested in Caco cells where dendrimer were conjugated with the drug and were able to retain the drug for the longer period of time in side the cell (Emanuele et al. 2004). The accumulation of the drug was achieved by three to four folds using this conjugation procedure (Carreno-Gomez and Duncan 2002). In the present case, PAMAM dendrimers also contribute to this as efflux of relatively large sized PAMAM dendrimers will be more difficult compared to drug alone.

Acquired resistance to chemotherapy is a major problem during cancer treatment. One mechanism for drug resistance is overexpression of the MDR (multidrug resistance) 1 gene encoding the transmembrane efflux pump, P-glycoprotein (P-gp) (Shiraki et al. 2001). Because of the P-gp the accumulation of many drugs inside the cells decreases and this effect is reversed by many P-gp inhibitors. Verapamil has been known to reverse cellular drug resistance and increase the accumulation of drugs inside the cells by inhibiting the P-gp (Bellamy et al. 1988). Verapamil blocks the efflux of many anticancer drugs like methotrexate and doxorubicin and leads to increased drug accumulation in the cell. Further experiments were carried out where the fluorescence intensity from HeLa 229 cell line was monitored in presence of verapamil and drugs (MTX and DOX) conjugated with PAMAM dendrimers. We have observed a further increase in the fluorescence intensity from the system in presence of verapamil (Fig. 5). The increase in fluorescence intensity may be due to the increase in the accumulation of these drugs in presence of verapamil and PAMAM dendrimer. This corroborates our hypothesis that the size of the drug carrier (PAMAM) plays an important role in reducing the rate of efflux and thereby increasing the local concentration of both PAMAM and the drug in the cell, providing an

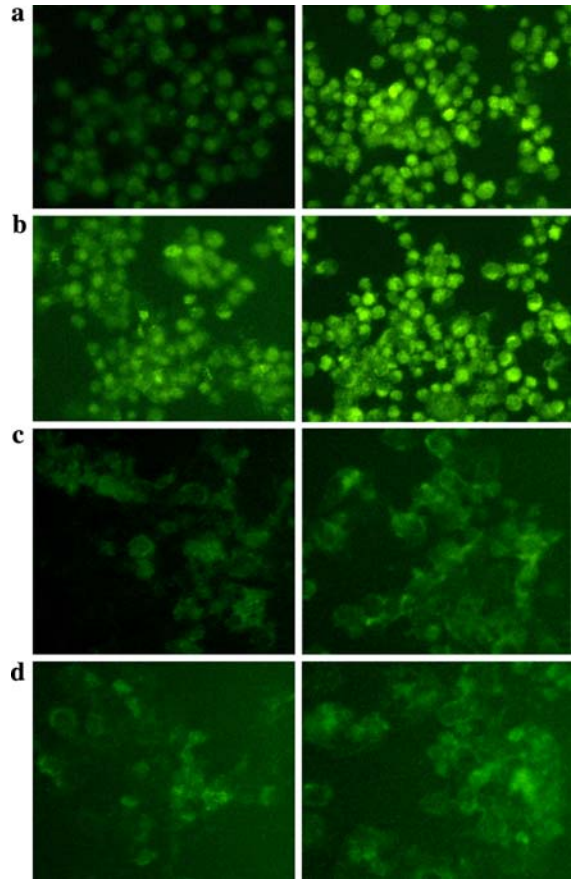

Fig. 5 Fluorescence images of HeLa 229 cell line treated with drugs alone or with verapamil; **a** DOX (10 μM) alone (*left*) and DOX+ Verapamil(10 μM) (*right*) **b** cells treated with DOX-PAMAM (10 μM) alone (*left*) and DOX-PAMAM+ Verapamil(10 μM) (*right*), **c** MTX (10 μM) alone (*left*) and MTX+ Verapamil (10 μM) **d** MTX-PAMAM (10 μM) alone (*left*) and MTX-PAMAM+ Verapamil (10 μM). All the experiments were done three times. The pictures shown were the representatives of three independent experiments

opportunity to image the cell through the intrinsic emission properties of the dendrimer and drug.

Conclusions

In summary, the experimental results presented here suggest that PAMAM-drug (MTX and DOX) complexes deliver MTX as well as DOX in a controlled way compared to the case where dendrimer is absent. The cytotoxicity studies suggested improved cell death in presence of dendrimer-drug combination, compared to control experiments with dendrimer or drug alone. Complex formation between the drugs and PAMAM dendrimer resulted in reduced efflux rate from the cell which in turn led to increased local concentrations of the dendrimer-drug complex inside the cell. As a result, the intrinsic emission from PAMAM dendrimer as well as the drug was enhanced inside the cell, which was effectively utilized to image HeLa 229 cells.

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