Preclinical antitumor efficacy evaluation of dendrimer-based methotrexate conjugates

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Our previous studies have demonstrated the in-vitro and in-vivo targeting of a generation-5 (G5) dendrimer-based multifunctional conjugate, which used folic acid (FA) as the targeting agent and methotrexate (MTX) as the chemotherapeutic drug. For the synthesized G5-FA-MTX nanodevice conjugate to be clinically applicable as a cancer therapeutic drug, it is important that the compound elicits cytotoxicity specifically and consistently. The aim of this work was to evaluate four independently synthesized batches of G5-FA-MTX conjugates for their cytotoxic potential and specificity. For determination of specificity, we have used a unique 'coculture' assay in which FA receptor-positive and FA receptor-negative cells were cultured together and have examined the preferential killing of the former. The results of our study show the batch-to-batch consistency and specificity of the G5-FA-MTX nanodevice in the preferential killing of FA receptor-positive cells. The coculture assay shows the consistency of the four different G5-FA-MTX conjugate lots in the specific killing of targeted cells. Further in-vivo studies are, however, necessary to prove the clinical potential of this targeted therapeutic nanodevice.
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Introduction

Traditional anticancer chemotherapy uses drugs that have minimal functional specificity for cancer cells [1]. As a result, anticancer drugs have extremely narrow therapeutic indices, which result in significant toxicity. A great need thus exists for anticancer drugs that specifically target cancer cells while leaving normal tissue intact.

We have recently reported dendrimer-based drug conjugates that target cancer cells through either the folate receptor [2–6], prostate specific membrane antigen [7], HER 2/*neu*, or the αvβ3 integrin [8]. For these 'targeted' drug delivery agents to be useful for clinical application, however, these compounds have to consistently show specific killing of the cells that have been targeted. Assaying the effects of anticancer agents on the in-vitro growth and survival of neoplastic cell populations has represented a critical tool in the preclinical evaluation of these therapeutics [9,10]. The most common techniques for testing anticancer drugs *in vitro* are proliferation assays such as the XTT or MTT assays [11,12] and the clonogenic assay, which assesses the number of cell colonies after exposure [13–16]. In addition, there are a variety of methods to measure apoptosis in cells after exposure to anticancer drugs. These include the flipping of the annexin V assay [17], measuring the activation of caspases [18–20], or labeling DNA strand breaks with 5-bromo-2′-deoxyuridine 5′-triphosphate [21]. All these assays, however, measure the effect of a drug on a single cell population; therefore, these assays do not necessarily reflect the specificity of the drug toward the targeted cell population. To simulate in-vivo conditions in which cancer cells can grow in the immediate vicinity of normal cells, we examined the specific killing of targeted cells cocultured with nontargeted cells. The coculture experiments have become more useful with the recent development of vectors expressing fluorescent proteins, which allow easy identification of different cell populations *in vitro* and *in vivo* [22–24]. It has thus become possible to grow two or more defined cell lines in coculture and to expose them simultaneously to anticancer agents, while monitoring the ability to define the cell populations. The advantage of testing the preferential cytotoxic potential of a drug using the coculture assay is that the effect of any diffusion of the drug from the targeted cells into the neighboring nontargeted cells (termed the 'bystander' effect) can also be inferred.

The aim of this study was the preclinical evaluation of the specific cytotoxicity of five independent batches of dendrimer-based methotrexate (MTX) conjugates that were designed and synthesized to target cancer cells via the folate receptor. We initially used the traditional assay methods to show the cytotoxicity of several independent generation-5–folic acid–MTX (G5-FA-MTX) batches. We then employed a coculture assay that consisted of two

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cell types: folate receptor-positive KB cells that express red fluorescent protein (RFP) and MCA-207 cells that lack the receptor and express green fluorescent protein (GFP). The results of our study confirm the consistency and specificity of the G5-FA-MTX dendrimer conjugate for targeted drug delivery in FA receptor-expressing cancer cells.

Materials and methods

Materials

Generation-7 nonacetylated dendrimers (G7-PAMAM) and one lot of G5 dendrimers onto which FA and MTX were attached (M269-29) were synthesized at Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan as previously described [3,25], and three other lots (123-134, 182-184, 06-02-0001) were manufactured by Cambrex Corporation (East Rutherford, New Jersey, USA). Free MTX was purchased from Sigma (St Louis, Missouri, USA). The plasmid pDsRed1-N1, which constitutively expresses RFP under the control of a $P_{CMV\ IE}$ promoter, was purchased from Clontech (Mountain View, California, USA).

Transfection of KB cells with pDsRed1-N1

Cell transfection was performed as previously described [26]. Briefly, KB cells were plated in six-well plates 18 h before transfection to achieve 60–70% confluency. G7-PAMAM and pDsRed1-N1 DNA were mixed in water at a charge ratio of 5 : 1. The dendrimer/DNA complex was then allowed to form for 15 min at room temperature. Cells were washed with serum-free medium, and 50 µl of the complex (1 µg DNA/well) was added to each well in 1 ml of serum-free medium and incubated for 3 h at 37°C, 5% CO_2. The medium containing the complex was then replaced with complete growth medium. The cells remained in culture for 24 h before selection, using G418 (200 µg/ml). Stable transfectants were established by growing cells in selective medium, and were cloned using the limiting dilution technique. Expression of the reporter gene was analyzed using fluorescence microscopy and flow cytometry.

Cell cultures

KB is a human epidermal carcinoma cell line (ATCC, Manassas, Virginia, USA) that overexpresses folate receptors, especially when grown in low-FA medium [27]. The KB cells were grown as a monolayer cell culture at 37°C and 5% CO_2 in FA-deficient RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 µl/ml), and 10% heat-inactivated fetal bovine serum. The MCA-207, a mouse sarcoma cell line stably transfected with GFP, was kindly provided by Dr Kevin McDonough at the University of Michigan. The original MCA-207 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-activated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µl/ml), and 2 µmol/l L-glutamine.

To perform coculture experiments, MCA-207/GFP and KB/RFP cells were mixed and grown to test whether both cells could grow together at the same rate as individual cell cultures and form a homogeneous monolayer. The estimated doubling times for the KB and MCA-207 cells were 16.4 and 14.1 h, respectively. To study the cytotoxic effect of the G5-FA-MTX conjugates in a coculture assay, approximately 7.5×10^4 KB and 2.5×10^4 MCA-207 were mixed and seeded/well of a six-well plate in FA-deficient medium (1 ml/well). One hour later, the cells were treated as described below.

XTT assay

For the cytotoxicity experiments, MCA-207 or KB cells (2×10^3/well) were seeded in 96-well microtiter plates in FA-deficient medium 24 h before treatment. One hour before treatment, the microtiter plates were washed with FA-deficient medium, and 100 µl of fresh medium was added to each well. The cells were treated either with free MTX or with G5-FA-MTX conjugate for 72 h. A colorimetric XTT assay from Roche Molecular Biochemicals (Indianapolis, Indiana, USA) was carried out by following the vendor's protocol. After incubation with an XTT-labeling mixture, microtiter plates were read on an enzyme-linked immunosorbent assay reader (Synergy HT, Winooski, Vermont, USA; BioTek, Winooski, Vermont, USA) at 492 nm with the reference wavelength at 690 nm. Vehicle-treated cells were assigned a value of 100%.

Determining the methotrexate cytotoxic dose of 50% using an XTT assay

The cytotoxic dose of MTX resulting in a 50% reduction of viable cells (CD_{50}) was determined by XTT assay for both cell lines. The CD_{50} was calculated by extrapolation of the corresponding dose–response curve on a log–linear plot employing the portions of the curve that transected the 50% response point. The CD_{50} (MTX) values were 31 nmol/l for the MCA-207 and 27 nmol/l for the KB cells, respectively.

Clonogenic assay

The clonogenic assay was performed with some modifications as described previously [13]. Briefly, cells at a density of 10^3 were cultured in 60-mm dishes in FA-deficient medium and with free MTX or a G5-FA-MTX conjugate (at 60 nmol/l of an equimolar concentration of MTX) for 7 days. After incubation, the cells were stained with methylene blue, and colonies consisting of at least 30 cells were counted using the AcuCount1000 (Bio Logics, Manassas, Virginia, USA) counter.

Treatment of the coculture with free methotrexate and generation-5–folic acid–methotrexate conjugate

The cells in the coculture were exposed either to free MTX or to G5-FA-MTX conjugates in FA-deficient medium for 24 h. The medium was then replaced

with fresh, complete medium (without treatment), and the cells were incubated for an additional 48 h. Before studying the targeting efficiency of G5-FA-MTX conjugates against KB cells in the coculture assay, we assessed the cytotoxic doses of 90% (CD_{90}) of the free MTX and the G5-FA-MTX conjugates, which were 300 nmol/l for the free MTX and 600 nmol/l for the G5-FA-MTX conjugates, respectively (data not shown).

Fluorescent microscopy and flow cytometric analysis

At the end of the coculture assay, the cells were photographed using fluorescent microscopy. The cells were then harvested, resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin, and acquired on a Beckman-Coulter EPICS-XL MCL flow cytometer. The data collected were analyzed using Expo32 software (Beckman-Coulter, Miami, Florida, USA). To estimate the total number of cells in each coculture, equal volume samples of the coculture were acquired and analyzed using flow cytometry.

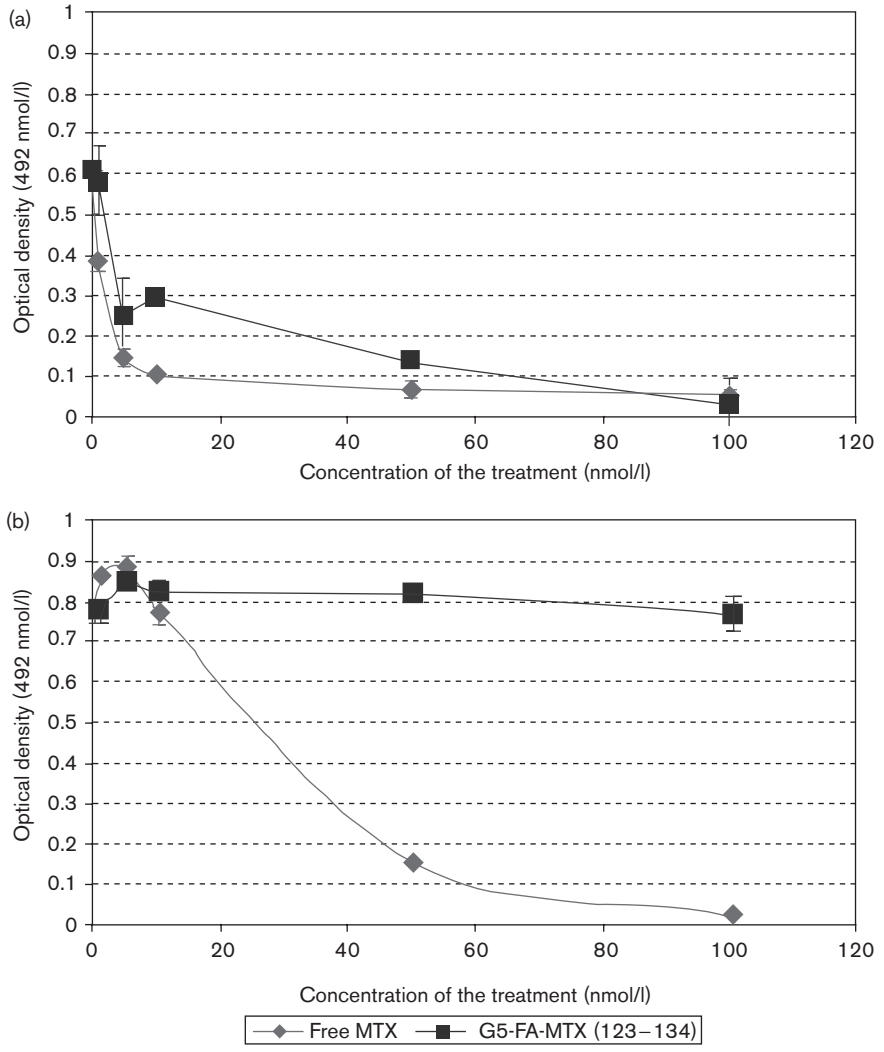
Statistics

All data are expressed as a mean of values and ± SEs. To assess the preferential cytotoxicity of the G5-FA-MTX conjugate, we calculated the odds ratio (OR) using the formula described by Hausner *et al.* [28]:

$$\mathrm{OR} = (T_t\%/(100 - T_t\%))/(T_c\%/(100 - T_c))$$

where $T_t\%$ is the percentage of the targeted cell population in the coculture exposed to the drug and

Fig. 1



Cytotoxicity of free MTX and the G5-FA-MTX conjugate against KB (a) and MCA-207 (b) cells as measured in an XTT assay. Cells were seeded in 96-well microtiter plates 24 h before treatment. An hour before treatment, cells were washed, 100 μl of fresh medium was added to each well and cells were treated either with free MTX or with G5-FA-MTX conjugate. Cells were incubated for 72 h. Afterward, the incubation plates were subjected to XTT colorimetric assay. G5-FA-MTX, generation-5–folic acid–methotrexate.

T_c% is the percentage of the targeted cell population in the control (not exposed to the drug) coculture.

Results

Cytotoxicity of methotrexate and the generation-5–folic acid–methotrexate conjugate against KB and MCA-207 cells as measured in an XTT assay

KB and MCA-207 cells were continuously treated either with free MTX or with the G5-FA-MTX conjugate (Lot 123–134) for 72 h, and the viability of the cells was measured in an XTT colorimetric assay. The free MTX was equally cytotoxic to both KB and MCA-207 cells (Fig. 1). In contrast, the G5-FA-MTX conjugate was cytotoxic to KB cells but displayed no cytotoxicity against MCA-207 cells, even at a concentration of 100 nmol/l (Fig. 1). Figure 2 compares the cytotoxicity of two different lots of the G5-FA-MTX conjugate against KB and MCA-207 cells. All tested lots of the G5-FA-MTX conjugate were cytotoxic to KB, but not to MCA-207 cells, at a concentration of 100 nmol/l.

Effect of generation-5–folic acid–methotrexate conjugate on KB and MCA-207 cells, as measured by using a clonogenic assay

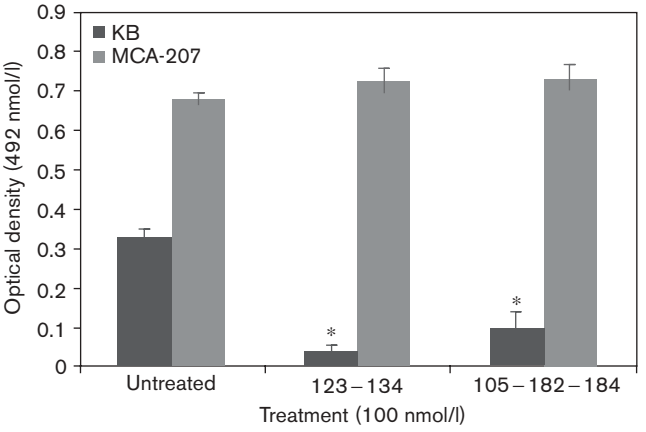
Two lots (123–134 and 182–184) of the G5-FA-MTX conjugate were tested to determine its cytotoxicity against KB and MCA-207 cells, using a clonogenic assay. Both lots of the G5-FA-MTX conjugate were cytotoxic to KB but not to MCA-207 cells (Fig. 3). These results further confirm the specificity of the G5-FA-MTX conjugate to target the folate receptor-positive KB cells.

Quantitative analysis of the cytotoxic specificity of the generation-5–folic acid–methotrexate conjugate, using cocultured KB and MCA-207 cells

To prove whether the G5-FA-MTX conjugate was specifically cytotoxic to the KB cells, cocultures of the KB and MCA-207 cells were designed. Cocultures of KB and MCA-207 cells were treated for 24 h with either free MTX or the G5-FA-MTX conjugate, Lot 123–134. The treatment was washed out, and the cocultures were incubated for an additional 48 h without treatment. Both KB and MCA-207 cells were uniformly killed by the free MTX, and the few remaining cells were rounded and loosely attached to the surface (Fig. 4b). In contrast, the G5-FA-MTX conjugate reduced the number of KB cells exclusively (compare lower panel with upper panel of Fig. 4c). Flow cytometry analysis confirmed and extended the microscopic observation. In the control cocultures, the cell ratio of KB to MCA-207 cells was 7:1, owing to the different growth rates of the two cell lines. In the cultures treated with the two lots of the G5-FA-MTX conjugate, the ratios were 1:1 and 1.8:1 for Lots 123–134 and 182–184, respectively, indicating that treatment with the G5-FA-MTX conjugate was preferentially cytotoxic to KB cells (Fig. 5). Using a formula described by Hausner *et al.* [28], the estimated ORs were 0.16 and 0.30 for Lots 123–134 and 182–184, respectively, indicating that the G5-FA-MTX conjugate specifically killed the KB cells versus the MCA-207 cells. For comparison, the OR calculated for the free MTX was 2.5.

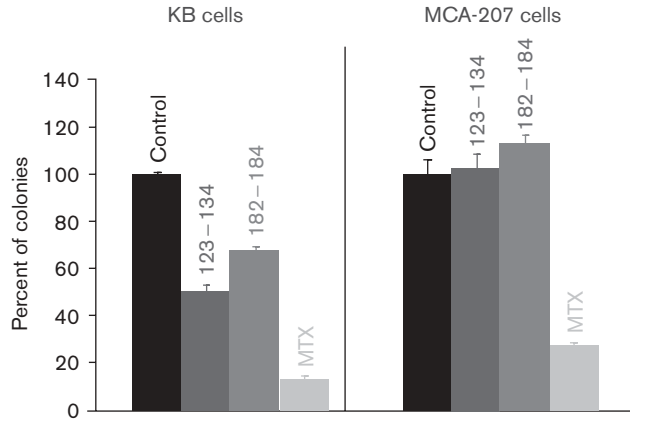
Although the OR indicated that the G5-FA-MTX conjugate had preferential cytotoxicity against KB cells, the OR itself did not specify the definite loss of either cell population caused by the treatment. To assess the

Fig. 2



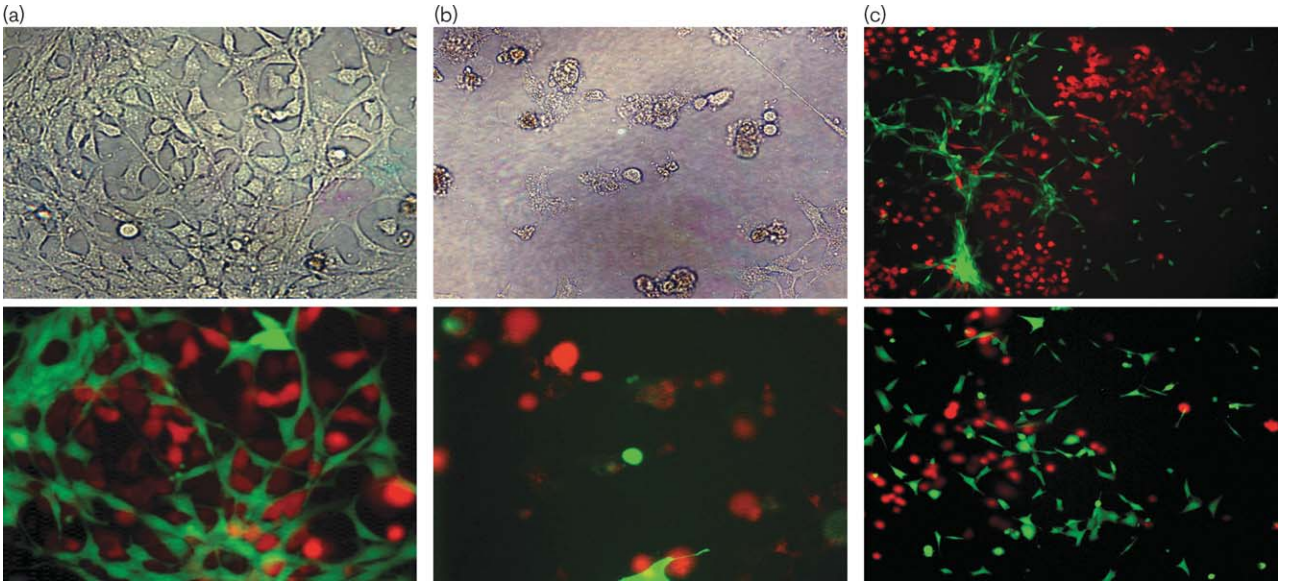
Cytotoxicity of two different lots of G5-FA-MTX conjugate (182–184, 123–134) is demonstrated with KB and MCA-207 cells using an XTT assay. The tested lots of G5-FA-MTX conjugate were cytotoxic to KB but not to MCA-207 cells at a concentration of 100 nmol/l. Asterisks indicate the significance between the treated and untreated (control) cultures. G5-FA-MTX, generation-5–folic acid–methotrexate.

Fig. 3



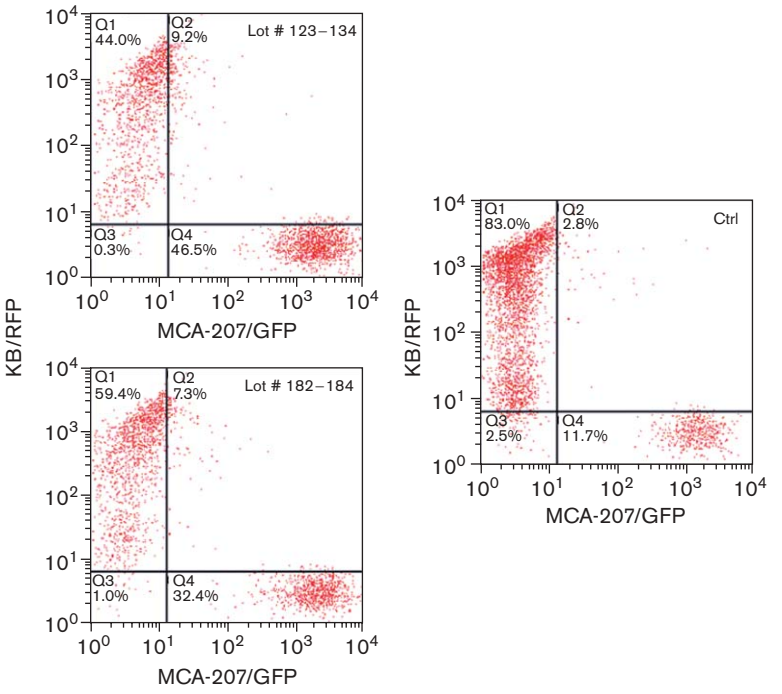
In-vitro cytotoxicity of two lots of the G5-FA-MTX conjugate (182–184, 123–134) against KB and MCA-207 cells, determined by a clonogenic assay. The cells cultured in 60-mm dishes were treated with 60 nmol/l of either free MTX or the G5-FA-MTX conjugate for 7 days with two changes of media, and the number of cell colonies was counted. Both the tested lots of G5-FA-MTX conjugate were cytotoxic to KB but not to MCA-207 cells. G5-FA-MTX, generation-5–folic acid–methotrexate.

Fig. 4



The MCA-207 (green) and KB (red) cells were untreated (a) and treated with free MTX (300 nmol/l) (b) or the G5-FA-MTX conjugate (600 nmol/l) (c, lower panel) for 24 h. The medium was then replaced with fresh complete medium (without treatment), and the cells were incubated for an additional 48 h. Upper panels of (a) and (b) are pictures taken in visible light. The treatment with free MTX killed both populations of cells. A few remaining cells were rounded and loosely attached to the surface (b). KB cells were reduced in number compared with the control culture (c, upper panel). G5-FA-MTX, generation-5–folic acid–methotrexate.

Fig. 5



Flow cytometry analysis of the KB and MCA-207 cells cocultured and treated with the G5-FA-MTX conjugate (Lots 123–134 and 182–184) as described in the Materials and methods section. In the control coculture, the ratio of KB cells to MCA-207 cells was 7 : 1. In the cultures treated with the G5-FA-MTX conjugate, the ratio was 1 : 1 for Lot 123–134 and 1.8 : 1 for Lot 182–184, respectively, indicating preferential cytotoxicity toward KB cells. G5-FA-MTX, generation-5–folic acid–methotrexate.

Table 1 Cytotoxic effect of free MTX and G5-FA-MTX conjugates on cocultured KB and MCA-207 cells[a]

	KB		MCA-207		
Treatment[b]	Number of cells/sample	SE	Number of cells/sample	SE	Targeting index[c]
Untreated	6858	140	23 152	1502	–
MTX	30	4	77	31	0.75
123–134	791	275	9100	3037	3.41
182–184	694	226	7777	1625	3.32
06-02-0001	1065	1	10 393	1743	2.89
M269-29	768	78	10 247	1611	3.95

[a]Approximately 5×10^4 KB-RFP and 5×10^4 MCA-207-GFP cells/well were mixed and seeded in the wells. One hour later cells were treated either with free MTX or MTX conjugates for 72 h. Then cells were harvested, resuspended in the original volume of flow cytometry buffer, and acquired on a Beckman-Coulter EPICS-XL MCL flow cytometer. To estimate the entire number of cells, equal cell suspension volumes for each coculture sample were acquired.
For more details see Materials and methods section.
[b]Cells were treated either with free MTX at a concentration of 300 nmol/l or conjugates at a concentration of 600 nmol/l.
[c]The targeting index is the fraction of surviving cells of the nontargeted population (MCA-207) divided by fraction of surviving cells of the targeted population (KB) and indicates the preferential killing of targeted cells over nontargeted cells.
GFP, green fluorescent protein; KB, human carcinoma cell line; MCA, mouse sarcoma cell line; MTX, methotrexate; RFP, red fluorescent protein.

total loss of cells in both populations, equal volumes of cell suspensions for each coculture sample were analyzed using flow cytometry. As shown in Table 1, the free MTX was equally cytotoxic to both cell populations, killing approximately 99% of the cells. Four different lots of the G5-FA-MTX conjugate were cytotoxic to various extents to the KB and MCA-207 cell populations. In all cases, the G5-FA-MTX conjugate showed preferential killing of the KB cells over the MCA-207 cells. On average, 88% of the KB cells versus 59% of the MCA-207 cells were killed by the tested lots of the G5-FA-MTX conjugate. Although the G5-FA-MTX conjugate killed significantly more targeted cells (KB), the ratio of targeted to nontargeted killed cells was approximately 3 : 1, which suggested some degree of nonspecific cytotoxicity for these conjugates that had not been observed in the earlier assays (Figs 1–3). This cytotoxicity might have occured owing to a bystander effect described for other drugs studied in a coculture system [29], and needs to be further investigated.

Discussion

Dendrimer-based drug-delivery molecules have several potential advantages. Dendrimers are comparable in size to proteins, being small enough (< 5.0 nm in diameter) to escape the vasculature and target tumor cells, and being below the threshold of renal filtration to allow urinary excretion. This latter asset forestalls retention in the filter organs; therefore it removes a requirement for hepatic metabolism. The G5 PAMAM dendrimer is stable and nonimmunogenic, and it contains on average 110–128 primary amines on its surface. Owing to this latter characteristic, it provides ample reactive sites for the conjugation of complex drug-delivery systems and multiple chemical moieties, such as radiopharmaceuticals, dyes, and contrast agents. Dendrimers can be chemically synthesized in large quantities as monodispersed populations, allowing for the potential scale-up of the technology.

Recently, we achieved significant improvements in the therapeutic index of a targeted MTX conjugate over the free drug, and this could have occurred from both a decrease in toxicity and an increase in drug effectiveness [2]. MTX conjugated to the dendrimer had a significantly lower toxicity and a 10-fold higher efficacy, compared with free MTX at an equal cumulative dose [2].

The results of these studies prompted us to work on the reproducible synthesis of targeted MTX conjugates. We have also demonstrated through chemical analysis the feasibility of achieving a reproducible synthesis of targeted drug conjugates [3]. Although chemical analysis of targeted drug conjugates confirmed that all lots are comparable, a simple and dependable method has to be applied to test the reproducibility of the biological effects of the drug. The methods commonly used measure the effect of a drug on a single cell population that does not necessarily reflect the specificity of the drug toward the targeted cell population. To this end, we applied a coculture assay, to test the effect of the drug on targeted and nontargeted cell populations simultaneously. Using the coculture assay, we could calculate the absolute number of cells in both populations. The estimation of the total number of cells in each population in a coculture offers important information on the interaction of the tested anticancer drugs with both targeted and nontargeted 'bystander' cells. This study demonstrates the potential applicability of a coculture assay for determining the specificity of a chemotherapeutic drug. In our assay, we limited the number of cell populations to two. More cell populations can, however, be used in a coculture assay to study the effect of a drug. The only limiting factor here is a method of distinguishing between the different cell populations that are in a culture together. It is relatively easy to improve the coculture assay by adding a third nonfluorescent monocytic or lymphocytic cell population, to study the effect of the immune response on an anticancer therapy *in vitro*. With the recent development of vectors expressing fluorescent

proteins, which allow for the easy identification of different cell populations, a more sophisticated coculture assay can be designed to supply more detailed information on the interaction of cell populations with each other and with the targeted drug *in vitro*. The aim of our study was to test whether reproducibility in the chemical synthesis of the targeted MTX conjugate relates to the reproducibility of the drug effect on targeted cells. Using the coculture method, we proved that all four tested lots of targeted MTX conjugate are comparable in dose and in their cytotoxic effects on targeted cells.

Conclusion

In this report, we have evaluated G5-FA-MTX conjugates that target MTX to KB cells via the folate receptor and have found them to be preferentially cytotoxic to KB cells. Analysis of the coculture assay demonstrated that, compared with MCA-207 cells, the KB cells were killed with a targeting index of 3.4. These findings prove the reproducibility of all tested G5-FA-MTX conjugate lots in the specific killing of targeted cells. Further in-vivo studies are, however, warranted, to prove the reproducibility of the targeted therapeutic nanodevice.

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