Polymeric nanogels containing the triphosphate form of cytotoxic nucleoside analogues show antitumor activity against breast and colorectal cancer cell lines

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Abstract
The therapeutic efficiency of anticancer nucleoside analogues (NA) strongly depends on their intracellular accumulation and conversion into 5'-triphosphates. Because active NATP cannot be directly administrated due to instability, we present here a strategy of nanoeapsulation of these active drugs for efficient delivery to tumors. Stable lyophilized formulations of 5'-triphosphates of cytarabine (araCTP), gemcitabine (dFdCTP), and floxuridine (FdUTP) encapsulated in biodegradable PEG-cl-PEI or F127-cl-PEI nanogel networks (NGC and NGM, respectively) were prepared by a self-assembly procedure. Cellular penetration, in vitro cytotoxicity, and drug-induced cell cycle perturbations of these nanofomulations were analyzed in breast and colorectal cancer cell lines. Cellular accumulation and NATP release from nanogel was studied by confocal microscopy and direct high-performance liquid chromatography analysis of cellular lysates. Antiproliferative effect of dFdCTP nanofomulations was evaluated in human breast carcinoma MCF7 xenograft animal model. Nanoencapsulated araCTP, dFdCTP, and FdUTP showed similar to NA cytotoxicity and cell cycle perturbations. Nanogels without drugs showed very low cytotoxicity, although NGM was more toxic than NGC. Treatment by NATP nanofomulations induced fast increase of free intracellular drug concentration. In human breast carcinoma MCF7 xenograft animal model, i.v. dFdCTP-nanogel was equally effective in inhibiting tumor growth at four times lower administered drug dose compared with free gemcitabine. Active triphosphates of NA encapsulated in nanogels exhibit similar cytotoxicity and cell cycle perturbations in vitro and faster cell accumulation and equal tumor growth-inhibitory activity in vivo at much lower dose compared with parental drugs, illustrating their therapeutic potential for cancer chemotherapy. [Mol Cancer Ther 2008;7(10):3373–80]

Introduction
Nucleoside analogues (NA) and nucleobases are widely used for the treatment of cancer (1). For instance, the pyrimidine analogue arabinofuranosylcytosine (cytarabine, araC) constitutes the cornerstone of induction therapy in patients with acute myeloid leukemia, whereas the fluorinated pyrimidine analogue gemcitabine (dFdC) has much broader indications, including lung, breast, pancreatic, and bladder cancers. Finally, the fluoropyrimidine 5-fluorouracil (5-FU) continues to be one of the most widely prescribed cancer chemotherapeutic drugs in the world. These drugs are administered as inactive prodrugs entering cells by means of specific membrane nucleoside transporters (2). Once inside the cell, these compounds can be activated by different nucleoside kinases to produce biologically active diphosphorylated and triphosphorylated metabolites (3). Thus, the therapeutic efficacy of NA strongly depends on its intracellular transport and phosphorylation. Any ways to increase the intracellular concentration of active phosphorylated drug metabolites would increase clinical efficacy of cancer chemotherapy. Negatively charged nucleoside 5'-mono-, di-, and triphosphates cannot permeate through the lipid-rich cellular membrane and are immediately degraded in the gastrointestinal tract or in the blood circulation (4). Several strategies including pronucleotide approach, liposomal encapsulation, nanoparticles or RBC-based delivery were evaluated previously to bypass the bottleneck in tumor accumulation of the activated drugs; unfortunately, none of them found its way to clinic (5). The pronucleotide approach was to administer protected or lipophilic prodrugs of nucleoside 5'-monophosphates (6–8). However, this strategy showed many disadvantages such as degradation of pronucleotides in serum or tumor microenvironment, production of charged phosphodiester intermediates resistant to further chemical degradation, and possible catabolism of 5'-monophosphates by 5'-nucleotidases (4). The second approach was to encapsulate the activated phosphorylated drugs in liposomes (9–12).
Different caveats were encountered when working with these liposomal drugs. The nucleotides could not diffuse readily through the intact liposome membrane affecting the in vivo activity of encapsulated drugs. It was found that the size, surface charge, and lipid composition had a strong influence on liposome clearance profile. In the third approach, 5'-mono- and triphosphates of NA were encapsulated in erythrocytes (13, 14). The major problem encountered in the use of natural cells as drug carriers was their efficient removal from blood circulation by the reticuloendothelial system.

We describe novel formulations of 5'-triphosphates of cytotoxic NA in nanogels that were capable to deliver and rapidly release these active drug metabolites inside cancer cells. The nanosized hydrophilic networks composed of cross-linked cationic and neutral polymers have been initially applied to encapsulation and delivery of oligonucleotides (15). Recently, Vinogradov et al. have shown successful encapsulation of 5'-triphosphates of cytotoxic and antiviral NA in various biodegradable cationic nanogels (16). Together with these encouraging results, several recent publications confirmed that direct administration of 5'-triphosphates encapsulated in nanoparticles could increase therapeutic efficacy of NA (17, 18). The aim of this study was to determine whether nanogel formulations can supply effective doses of active NATP into various cancer cells. Here, we report that biodegradable nanogels (denoted as NGC and NGM) loaded with NATP of araC, FdU, and dFdC (Supplementary Fig. S1B)3 were converted into 5'-triphosphates using the one-pot phosphorylation protocol with minor modifications (21). NATP were purified by the two-step procedure using a short column with Sephadex A-25 (acetate form) equilibrated with 0.05 mol/L TBAA (pH 6). The product was eluted from the column with a TBAA gradient from 0.05 to 1.5 mol/L. The NATP-containing fractions were usually contaminated with coeluted inorganic pyrophosphate; therefore, they were collected and applied directly into a short column with Silicagel C18 for desalting and eluting using a methanol gradient from 5% to 70%. Concentrated in vacuo NATP was precipitated in 1% sodium perchlorate in acetone as sodium salt. The purity of NATP products was ≥90% according to ion-pair reverse-phase high-performance liquid chromatography and UV spectrophotometric analysis. Complete synthetic procedure and characterization of the nucleoside 5'-triphosphates will be reported elsewhere.

**Materials and Methods**

**Materials**

All chemical reagents and solvents, if not mentioned otherwise, were purchased from Sigma-Aldrich. Pluronic F127 was generous gift from BASF, araC was purchased from 3B Medical Systems, FdU was from SynQuest Laboratories, and ATP BODIPY FL was from Invitrogen/Molecular Probes.

**Nanogel Preparation**

Nanogel NGC carrier representing a chemically cross-linked network of PEG molecules and biodegradable segmented PEI connected by disulfide bridges (PEG-cl-PEI) was synthesized by the emulsification-solvent evaporation method (ref. 19; Supplementary Fig. S1A).3 Nanogel NGM carrier contains lipophilic polymer core of Pluronic F127 surrounded by the covalently cross-linked network of PEG and biodegradable PEI prepared using micellar approach (20). Both nanogels have been fractionated by preparative SEC on Sephacryl S-300 column to isolate particles with hydrodynamic diameter in the range of 70 to 150 nm, neutralized with hydrochloric acid and lyophilized. Analytical characteristics of nanogels and drug nanoformulations are presented in Table 1. Rhodamine-labeled nanogels were prepared according to the previously described protocol (21).

**NATP Synthesis**

araC, FdU, and dFdC (Supplementary Fig. S1B)3 were converted into 5'-triphosphates using the one-pot phosphorylation protocol with minor modifications (21). NATP were purified by the two-step procedure using a short column with Sephadex A-25 (acetate form) equilibrated with 0.05 mol/L TBAA (pH 6). The product was eluted from the column with a TBAA gradient from 0.05 to 1.5 mol/L. The NATP-containing fractions were usually contaminated with coeluted inorganic pyrophosphate; therefore, they were collected and applied directly into a short column with Silicagel C18 for desalting and eluting using a methanol gradient from 5% to 70%. Concentrated in vacuo NATP was precipitated in 1% sodium perchlorate in acetone as sodium salt. The purity of NATP products was ≥90% according to ion-pair reverse-phase high-performance liquid chromatography and UV spectrophotometric analysis. Complete synthetic procedure and characterization of the nucleoside 5'-triphosphates will be reported elsewhere.

**Drug Encapsulation and Nanoformulation Stability**

The following protocol was used for drug encapsulation into nanogels (Fig. 1). Equal volumes of aqueous solutions of nanogel (40 mg/mL) and NATP (10 mg/mL) in water were mixed together and incubated for 30 min on ice. The mixture was applied into NAP-25 cartridge and the high molecular weight fraction was eluted with water and

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3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Table 1. Analytical characteristics of cationic nanogels and NATP nanoformulations

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Nitrogen content (µmol/mg)*</th>
<th>Nanogel diameter (nm)†</th>
<th>Drug loading (µg/mg nanoformulation)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unloaded</td>
<td>CTP-loaded</td>
</tr>
<tr>
<td>NGC</td>
<td>6.1</td>
<td>100 ± 8</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>NGM</td>
<td>5.5</td>
<td>187 ± 6</td>
<td>112 ± 3</td>
</tr>
</tbody>
</table>

*Calculated based on elemental analysis data.
†Measured by dynamic light scattering at 10 or 1 mg/mL with CTP (PBS; mean ± SD; n = 3).
§Determined spectrophotometrically in water using extinction coefficients for corresponding NA.
lyophilized. Nucleoside content in nanogels (Table 1) was detected spectrophotometrically using extinction coefficient $\varepsilon (\lambda_{max})$ values for dFdC: 9,360 (268 nm), FdU: 7,570 (268 nm), araC: 9,260 (272.5 nm), and cytidine: 9,100 (271 nm; ref. 22).

Stability of the drug nanoformulations in the presence of serum was evaluated by analytical SEC on Sephacryl S-500 column (1 x 25 cm) with UV detection at 280 nm in PBS. NGC nanogel was complexed with ATP as described above, mixed with equal volume of 10% serum, and analyzed immediately or following 90 min incubation at 37°C (Supplementary Fig. S2).

**Cell Lines**

The breast cancer cell lines MCF7 and T47D and the colorectal cancer cell lines HCT116, SW48, and SW480, all purchased from the American Type Culture Collection, were cultured in DMEM containing 10% FCS, 1% L-glutamine, and 2% penicillin/streptomycin on 25 cm$^2$ flasks at 37°C in a humidified atmosphere containing 5% CO$_2$.

**Nanogel Internalization Assays**

Rhodamine-labeled nanogel internalization assay was conducted in MCF7 and HCT116 cancer cells for 1 h at 37°C. Samples were trypsinized, washed twice in PBS, and acquired in a FACSCalibur flow cytometer (Becton Dickinson). Intracellular fluorescence analysis was done with FlowJo 7.2.2 software (Treestar).

Cellular accumulation and drug release was also examined by confocal microscopy in live MCF7 cells without fixation using a formulation of rhodamine-labeled nanogel NGC with the fluorescent ATP BODIPY FL prepared as described above. MCF7 cells were plated at a density of 50,000 per plate (Bioptechs) and allowed to attach for 24 h before the treatment. Cells were incubated with 1 mL of 0.001% drug-nanogel formulations for 1 h at 37°C and washed three times with ice-cold PBS containing 1% BSA, and the intracellular trafficking of nanogel and ATP release were investigated at two fluorescence modes for BODIPY and rhodamine dye ($\lambda_{ex}$ 549 nm/$\lambda_{em}$ 577 nm) using Zeiss confocal LSM410 microscope equipped with argon-krypton laser.

**dFdC Internalization**

Analysis of dFdCTP in the total NTP pool was done using the modified method (23). MCF7 cells (2 x 10$^6$) were seeded in six-well plate and treated with dFdC or NGC-dFdCTP formulation in full medium (1 µmol/well) for 2 and 6 h at 37°C. Two identical plates were used as parallels. Cells were trypsinized, collected at 400 x g (10 min, 4°C), washed, and counted. Cells resuspended in 240 µL water were treated with 80 µL of 40% TCA and placed on ice for 20 min, allowing protein and nucleic acids precipitation. After centrifugation at 16,000 x g (10 min, 4°C), supernatants were collected and extracted three times with 640 µL freshly prepared trioctylamine/freon (1,1,2-trichlorotrifluorethane) mixture (1:4, v/v). The aqueous part was analyzed by the ion-pair high-performance liquid chromatography. The 250 µL samples were injected on Zorbax C18 analytical column (100 x 4.6 mm, 3 µm particle size), and dFdCTP was separated from other cellular triphosphates in a gradient from 35% to 85% B (30 min) using solutions of (A) 150 mmol/L KH$_2$PO$_4$/10 mmol/L TBAH (pH 6.1) and (B) 150 mmol/L KH$_2$PO$_4$/10 mmol/L TBAH (pH 5)/20% methanol. Data were analyzed using the calibration curve for dFdCTP sample and recalculated according to the total sample volume and cell number; the final dFdCTP nucleotide content was expressed as pmol/10$^6$ cells.

**In vitro Growth-Inhibitory Assay**

Cell growth inhibition was determined using the MTT assay as described previously (24). Chemosensitivity was expressed as the effective drug concentration that inhibited cell proliferation by 50% (IC$_{50}$ values) and was determined from concentration-effect curves generated using Prism 4 software (GraphPad).

**Cell Cycle Distribution Analysis**

For analysis of DNA content and cell cycle distribution, MCF7 and HCT116 cells were treated with cytotoxic NATP-loaded nanogels, nontoxic CTP-loaded nanogels, or NA drugs for 24 h at 37°C. After drug exposure, 10$^6$ cells were resuspended in 2 mL propidium iodide solution (50 µL/mL), incubated for 1 h at 4°C, and analyzed using FACSCalibur flow cytometer (Becton Dickinson). Cell

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**Figure 1.** Formulation of nanogels with 5'-triphosphates of NA. **A**, self-assembly of nanoformulations with NATP. **B**, transmission electron microscopy of the drug-loaded nanocarrier negatively contrasted by vanadate. Bar, 100 nm.
cycle distribution and DNA ploidy status were calculated after exclusion of cell doublets and aggregates on a FL2-area/FL2-width dot plot using FlowJo 7.2.2 software (Treestar).

**In vivo Tumor Growth Inhibition Assay**

The human breast carcinoma MCF7 cell line was used to determine an in vivo tumor growth inhibition effect of the dFdC nanoformulation. A single-cell suspension of 5 × 10^6 cells in 400 μL full medium containing 20% Matrigel (Becton Dickinson) was s.c. injected into mammary fat pads of female NIH-III mice (Charles River Laboratories) ages 6 to 8 weeks. Animal studies were carried out according to the Institutional Animal Care and Use Committee guidelines. Mice were monitored daily for tumor growth, which appeared ~3 weeks after the cell injection. Animals were randomly divided into groups (n = 5) and received injections of PBS solution (control group) and well-tolerated doses of dFdC (6 mg/kg), dFdCTP/NGC (36 mg/kg; contains 3 mg/kg dFdCTP), and CTP/NGC (36 mg/kg; contains 4 mg/kg CTP) in the tail vein twice a week. The tumor volume was calculated based on the equation: TV = L / 2 × W^2, where L and W are length and width of tumor (mm) measured by calipers.

**Results**

**Drug Nanoformulations Stability and Cellular Uptake**

NGC and NGM nanogels schematic network and structures of 5'-triphosphates of NA are shown in Supplementary Fig. S1. These anionic drug derivatives were encapsulated in the positively charged nanogel network by simple mixing of both components in aqueous solution. The compact particles of drug-loaded nanogels with diameters 60 to 110 nm contained up to 19% weight of active drug and could be stored in lyophilized form (Table 1).

Stability and release of nanogel-encapsulated NTP were analyzed by analytic SEC following the incubation with serum-containing medium. As shown in Supplementary Fig. S2, the SEC profiles of ATP-loaded nanogel mixed with 10% serum were similar for the two conditions tested: injected immediately (Fig. 2C) or after incubation at 37°C for 90 min (Fig. 2D). Compared with the ATP-loaded nanogel injected without serum (Fig. 2A), both samples showed a minor additional peak eluting in the position of low molecular weight ATP and its metabolites. Serum (2%; Fig. 2B) had only a background absorbance as well as nonloaded nanogel (data not shown). Thus, incubation at 37°C in serum did not affect significantly stability of the nanoformulations.

To study internalization of nanocarriers, MCF7 and HCT116 cancer cells were incubated with rhodamine-labeled NGC or NGM nanogels loaded with CTP. Flow cytometry studies revealed high intracellular levels of both nanocarriers in MCF7 and HCT116 cancer cells (Fig. 2A). These data suggest that NGC or NGM nanocarriers are capable to deliver NATP drugs into cancer cells.

An efficient and rapid drug release from NTP-loaded nanogels was shown by confocal microscopy in MCF7 cells incubated for 1 h with rhodamine-labeled NGC.

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**Figure 2.** Cellular uptake of cationic nanogels and encapsulated drugs and intracellular drug release from nanocarriers. A, MCF7 and HCT116 cancer cells were incubated with 1 μg/mL rhodamine-labeled NGC (dotted line) or NGM (dashed line) nanogels for 1 h and intracellular fluorescence was detected by flow cytometry. Continuous line, control, nontreated cells. B, confocal microimage of MCF7 cells treated with 1.5 μg/mL ATP BODIPY FL (I) or 10 μg/mL nanogel formulation containing 1.5 μg/mL ATP BODIPY FL (II) for 1 h at 37°C. Nanogel (NGC) was labeled by rhodamine isothiocyanate. Magnification, ×100. C, intracellular dFdCTP levels (nmol/10^6 cells) determined by analytical ion-pair high-performance liquid chromatography following the treatment of MCF7 cells with 1 μmol dFdC or equivalent amount of NGC-loaded dFdCTP for 2 and 6 h.
encapsulating the fluorescent ATP BODIPY FL. As shown in Fig. 2B, the significant accumulation of drug-loaded nanogel (red and yellow fluorescence) was immediately accompanied by rapid appearance of high intracellular level of released ATP (green fluorescence). Following the accumulation of nanogels on the cellular membrane (red fluorescence), the major part of the released ATP was distributed in the cytoplasm already after 1 h incubation (green diffused fluorescence), whereas some amount of drugs remained encapsulated in nanogels (yellow fluorescence) or enclosed in endosomes (green dotted fluorescence).

Direct measurement of the accumulated dFdCTP level was done by high-performance liquid chromatography analysis of the total cellular NTP pool after treatment of MCF7 cells with free dFdC or dFdCTP-loaded NGC nanogel. The level of dFdCTP in nanogel-treated cells was near four times higher than the de novo dFdCTP content in dFdC-treated cells 2 h post-incubation (Fig. 2C). Interestingly, ~5-fold increase in the concentration of intracellularly synthesized dFdCTP was detected in MCF7 cells treated with dFdC 6 h post-incubation. However, this amount accounted only for 60% of the level of dFdCTP released from nanogel at this time point.

**In vitro Growth-Inhibitory Assays**

Cytotoxicity of araCTP-, FdUTP-, or dFdCTP-loaded NGC and NGM nanocarriers was compared with CTP-loaded or nonloaded nanogels in MTT assay. As shown in Table 2, an increased cytotoxic effect on breast (MCF7 and T47D) and colorectal (HCT116, SW480, and SW48) cancer cells was generally observed when drug-loaded nanogels were compared with nonloaded nanocarriers. With all NATP-loaded NGC formulations, IC_{50} values were lowered by 36- to 233-fold, 44- to 126-fold, 36- to 330-fold, 12-fold, 20- to 52-fold, and 4- to 15-fold for MCF7, T47D, HCT116, SW480, and SW48, respectively. NGC and NGM nanogels loaded with CTP were even less toxic than nonloaded nanocarriers; the observed increase in cytotoxicity was more profound compared with nonloaded nanocarriers. NGC was distinctly less toxic than NGM in free and CTP-loaded forms in all experiments; it was chosen for cellular uptake and tumor growth inhibition experiments.

As shown in Table 2, drug-loaded nanogels showed high cytotoxic activities in all studied cells with IC_{50} values in the nanomolar range or, in some cases, lower than 5 μmol/L. Parental drugs showed IC_{50} values in a similar range. No major differences were observed between NGC- and NGM-loaded nanocarriers. araCTP- and dFdCTP-loaded nanocarriers showed equal or slightly increased IC_{50} values compared with araC and dFdC. FdUTP-containing nanocarriers also showed equal or slightly elevated IC_{50} values compared with 5-FU; however, in SW480 and HCT116 cells, their cytotoxic activity was greater than that observed with 5-FU. These data indicate that NGC and NGM nanogels encapsulating cytotoxic NATP have similar cytotoxic activities compared with parental drugs.

**Cell Cycle Distribution Analysis**

NATP-loaded nanocarriers provoked similar to the parental drug-induced cell cycle perturbations in breast and colorectal cancer cells. As shown in Table 3, araC treatment induced a substantial accumulation of MCF7 and HCT116 in S phase. This accumulation occurred mostly at the expense of the G_{S}G_{1} fraction. Similar features were observed when both cell lines were exposed to NGC-araCTP or NGM-araCTP. However, the level of S-phase

<table>
<thead>
<tr>
<th>Drug (μmol/L)*</th>
<th>MCF7</th>
<th>T47D</th>
<th>SW480</th>
<th>SW48</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>araC</td>
<td>0.44 ± 0.1</td>
<td>0.1 ± 0.03</td>
<td>1.7 ± 0.7</td>
<td>0.04 ± 0.02</td>
<td>0.02 ± 0.008</td>
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<tr>
<td>NGC-araCTP</td>
<td>1.1 ± 0.5</td>
<td>0.35 ± 0.07</td>
<td>1.5 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>NGM-araCTP</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>1.5 ± 0.8</td>
<td>0.2 ± 0.08</td>
<td>0.2 ± 0.04</td>
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<tr>
<td>5-FU</td>
<td>0.36 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 1.5</td>
<td>6.5 ± 3</td>
<td>2.6 ± 0.8</td>
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<tr>
<td>NGC-FdUTP</td>
<td>1.9 ± 0.6</td>
<td>0.4 ± 0.1</td>
<td>1.7 ± 0.3</td>
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<td>0.6 ± 0.2</td>
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<td>NGM-FdUTP</td>
<td>2.4 ± 1</td>
<td>0.9 ± 0.3</td>
<td>4 ± 1</td>
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<tr>
<td>dFdC</td>
<td>0.009 ± 0.002</td>
<td>0.002 ± 0.001</td>
<td>0.01 ± 0.006</td>
<td>0.006 ± 0.002</td>
<td>0.005 ± 0.002</td>
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<td>NGC-dFdCTP</td>
<td>0.08 ± 0.02</td>
<td>0.14 ± 0.04</td>
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<td>0.25 ± 0.07</td>
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<tr>
<td>NGM-dFdCTP</td>
<td>0.06 ± 0.02</td>
<td>0.11 ± 0.05</td>
<td>0.22 ± 0.02</td>
<td>0.24 ± 0.06</td>
<td>0.07 ± 0.007</td>
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<table>
<thead>
<tr>
<th>Nanogel (μg/mL)</th>
<th>MCF7</th>
<th>T47D</th>
<th>SW480</th>
<th>SW48</th>
<th>HCT116</th>
</tr>
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<tbody>
<tr>
<td>NGC</td>
<td>210 ± 40</td>
<td>76 ± 7</td>
<td>330 ± 200</td>
<td>600 ± 100</td>
<td>400 ± 40</td>
</tr>
<tr>
<td>NGC-CTP</td>
<td>1160 ± 400</td>
<td>650 ± 10</td>
<td>750 ± 110</td>
<td>810 ± 20</td>
<td>630 ± 40</td>
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<tr>
<td>NGM</td>
<td>30 ± 10</td>
<td>20 ± 9</td>
<td>10 ± 2</td>
<td>100 ± 60</td>
<td>30 ± 8</td>
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<tr>
<td>NGM-CTP</td>
<td>59 ± 6</td>
<td>39 ± 20</td>
<td>54 ± 8</td>
<td>120 ± 23</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

*The drug concentration was calculated based on the drug content in nanogels and molecular weight of NATP.

*IC_{50} values represent mean ± SD of three separate experiments.
Table 3. Cell cycle distribution after nanogel-drug treatment by flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>MCF7</th>
<th>HCT116</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G0-G1</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>44 ± 7</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>araC</td>
<td>8 ± 4</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>NGC-araCTP</td>
<td>33 ± 8</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>NGM-araCTP</td>
<td>1 ± 1</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>5-FU</td>
<td>48 ± 4</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>NGC-FdUTP</td>
<td>43 ± 2</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>NGM-FdUTP</td>
<td>42 ± 2</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>dFdC</td>
<td>64 ± 7</td>
<td>34 ± 9</td>
</tr>
<tr>
<td>NGC-dFdCTP</td>
<td>50 ± 6</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>NGM-dFdCTP</td>
<td>50 ± 8</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>NGC-CTP</td>
<td>51 ± 3</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>NGM-CTP</td>
<td>51 ± 3</td>
<td>41 ± 5</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with 1 μmol/L of each compound for 24 h and analyzed. IC50 values represent mean ± SD of two separate experiments.

arrest was lower in HCT116 cells exposed to NGM-araCTP. Treatment of MCF7 cells with 5-FU induced a slightly increase of the G0-G1 fraction, whereas, in HCT116 cells, the drug mainly induced accumulation in S phase. In both cases, these alterations were produced at the expense of the G2-M cell fraction. In contrast, MCF7 and HCT116 cells treated with NGC-FdUTP and NGM-FdUTP showed a significant increase in G2-M cell fraction. In HCT116 cells, these compounds simultaneously induced an accumulation of cells in the S phase.

dFdC treatment induced accumulation of MCF7 cells in G0-G1 fraction associated with a reduction of cells within the S and G2-M phases. Instead, NGC-dFdCTP and NGM-dFdCTP nanocarriers induced not only a slight arrest at the G0-G1 fraction but also an accumulation in S phase. Likewise, dFdC-treated HCT116 cells arrested the cell cycle in S phase at the expense of the G2-M fraction. Similar features were observed when this cell line was treated with NGC-dFdCTP and NGM-dFdCTP nanofomulations. Thus, all these results show that, although NATP-loaded nanogels sometimes induced similar cell cycle perturbations compared with the parental drugs, they showed also some distinct nanocarrier-specific features.

In vivo Tumor Growth Inhibition Assay

The tumor growth inhibition following multiple i.v. administration of dFdCTP-loaded NGC nanogel was studied in the human breast carcinoma MCF7 xenograft mouse model. Median tumor volume changes in control and treated mice are shown in Fig. 3. Compared with control group, administration of dFdC in close to therapeutic dose induced a significant tumor growth inhibition and reduction. Injection of the well-tolerated dose of dFdCTP-NGC (36 mg/kg) showed statistically similar to dFdC-inhibitory effect (P = 0.096). However, the effective molar drug concentration in this formulation was four times lower than the equally effective dose of dFdC. CTP-nanogel showed some effect that was not, however, statistically significant compared with the control group. Statistically significant (P < 0.0001) differences were observed between the control group and the groups including dFdC- and dFdCTP-NGC-treated animals. Thus, NGC nanocarrier could efficiently deliver cytotoxic NATP to MCF7 xenograft tumors following systemic administration and showed comparable dFdC activity at significantly lower dose. There was no significant body weight loss among the groups throughout the experiment.

Statistical analysis was done by applying two-tailed unpaired t test (differences with P < 0.05 were considered significant) and F test to compare variances in groups using the Prism 4 software (GraphPad). The following P values were obtained: 0.002 (PBS/dFdC), 0.368 (PBS/CTP-NGC), <0.0001 (PBS/dFdCTP-NGC), 0.096 (dFdC/dFdCTP-NGC), and 0.0003 (dFdCTP-NGC/CTP-NGC).

Discussion

Development of novel tumor-targeted chemotherapies is required to achieve better drug accumulation in tumors and to overcome drug resistance to commonly used anticancer drugs. Many i.v. polymeric drug formulations were found to efficiently accumulate in vascularized tumors through their leaky blood vessels owing to the “enhanced permeability and retention” effect (25). We describe here application of hydrophilic polymeric nanogels for delivery of anticancer cytotoxic NA in their active 5′-triphosphate form. Our results show that cationic nanogels effectively encapsulate, deliver, and release NATP of araC, FdU, and dFdC inside breast and colorectal cancer cell lines inducing an in vitro cytotoxic activity and cell cycle perturbations similar to those observed for parental drugs. In some cases, these cell cycle alterations were specific for drug-loaded nanogel formulations, although nanogels by themselves showed an extremely low cytotoxicity. Rapid drug release was detected immediately following the nanogel internalization that could substantially
able buildup of nanogels on the surface of cellular
phosphorylated drug molecules. We observed a consider-
 uptake of nanogel-loaded NTP and sustained release of
to 40% (6 h; Fig. 2C). Our data showed an efficient cellular
efficient enough to reduce the difference from 300% (2 h)
de novo
tions of dFdCTP in the pool. Only at later time
administration, the disulfide bonds of nanogels rapidly degrade in the
presence of cytoplasmic glutathione into nontoxic PEG-g-
PEI conjugates having molecular weight below the kidney
excretion limit. In vitro NATP drug release from nanogel is
initially rather fast, accounting for 60% to 70% during the
first 24 h and the remaining 30% to 40% during the next
48 h (16). An approximately two-thirds of NATP was
released from nanogel during cytotoxicity assays, exhibiting
similar to NA cytotoxic effect in breast and colorectal cancer
cells. A direct evidence of higher dFdCTP-NGC accumula-
tion in the MCF7 cells was obtained by the extraction of total
cellular NTP/NATP and high-performance liquid chroma-
tography analysis of dFdCTP in the pool. Only at later time
points, de novo synthesis of dFdCTP from dFdC was
efficient enough to reduce the difference from 300% (2 h)
to 40% (6 h; Fig. 2C). Our data showed an efficient cellular
uptake of nanogel-loaded NTP and sustained release of
phosphorylated drug molecules. We observed a consider-
able buildup of nanogels on the surface of cellular
membrane and in endosomal compartments. This accumu-
lation was accompanied by a significant drug release in the
cytoplasm. Our previous studies showed that the drug
release can be mediated and enhanced by interaction with
 cellular membrane components (21). Although the mecha-

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Figure 3. Tumor growth inhibition exerted by active drug nanofor-
umulations of NA. Animal groups (n = 5) with developed human breast
carcinoma xenograft (MCF7) tumors were injected twice a week with
dFdCTP-NGC formulation (38 mg/kg, 6 μmol/L drug) or free dFdC (6 mg/kg,
23 μmol/L drug). PBS and an equivalent amount of CTP-NGC were used as
controls.
caveat is that drug nanoformulations could be as active in normal cells as in tumor. This problem could be solved by incorporating tumor-targeting ligands and site-specific release mechanism into nanogels, for example, by coating nanogels with peptides or antibodies that target over-expressed receptors or specific epitopes on the surface of tumor cells.

In summary, we showed that drug-loaded nanogels could deliver the active triphosphates of therapeutic NA into breast and colorectal cancer cells inducing an in vitro cytotoxic activity similar to that observed with the parental drug. Besides, the triphosphate-loaded nanoformulations exhibited a higher activity than NA drugs in animal models. These results illustrate the therapeutic potential of activated NA drugs formulated in nanosized biodegradable polymeric carriers for cancer chemotherapy. Given the potency observed with these drug nanoformulations, their application would resolve many of the problems associated with NA chemotherapy.

Disclosure of Potential Conflicts of Interest

S.V.V. is a shareholder of Supratek Pharma, the owner of Nanogel patent. No other potential conflicts of interest were disclosed.

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Polymeric nanogels containing the triphosphate form of cytotoxic nucleoside analogues show antitumor activity against breast and colorectal cancer cell lines

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