Bioorganic & Medicinal Chemistry Letters 19 (2009) 3725–3728

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Development of enzymatically cleavable prodrugs derived from dendritic polyglycerol

Marcelo Calderón [a], Ralph Graeser [b], Felix Kratz [c,*], Rainer Haag [a,*]

[a] Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany
[b] Proquinase, Breisacher Strasse 117, 79106 Freiburg, Germany
[c] Tumor Biology Center, Breisacher Strasse 117, 79106 Freiburg, Germany

ARTICLE INFO

Article history:
Received 16 February 2009
Revised 13 May 2009
Accepted 14 May 2009
Available online 18 May 2009

Keywords:
Polyglycerol
Polymer therapeutics
Prodrug
Doxorubicin
Methotrexate
Drug conjugate
Controlled release
Nanocarrier

ABSTRACT

In this Letter we report the synthesis and in vitro studies of cleavable polymer–drug conjugates derived from dendritic polyglycerol and maleimide-bearing prodrugs of doxorubicin and methotrexate that are cleaved by cathepsin B. Cleavage properties and cytotoxicity of the new conjugates are presented.

Many active molecules used in biomedical applications, for example, anticancer drugs, imaging probes, genes, etc., present a lack of specificity due to their low molecular weights which results in a uniform biodistribution, low concentration at the site of action, rapid plasma clearance, and high potential toxicity. These limitations can be overcome by the design of new macromolecular carrier systems. Several strategies have been pursued, that include active and passive targeting approaches with antibodies, serum proteins, liposomes, and synthetic polymers.[1–4]

Combination of active molecules with polymers may reduce their toxicity, eliminate undesirable body interactions, improve their solubility, bioavailability, stability, and prolong blood clearance.[5] Moreover, suitable polymer-based drug delivery systems can enable controlled release and specific delivery of bioactive agents to the diseased or damaged tissue (by non-covalent or covalent attachments).

Although significant efforts are being made to develop novel polymeric carriers, synthetic polymers which have been used in clinically evaluated drug conjugates are mainly restricted to *N*-(2-hydroxypropyl) methacrylamide copolymer (HPMA), polyethylene glycol (PEG), and poly(glutamic acid).[6] In addition, albumin, as a biopolymeric carrier, has been evaluated as a drug delivery system in anticancer therapy.[7]

In the design of new macromolecular carrier systems there is a great need for polymers that are highly water-soluble, nontoxic, nonimmunogenic, and biodegradable with low polydispersity and well-defined functional groups.[8] In this regard, dendritic polymers possess unique features that may be advantageous for drug delivery.[9–12] They can be tailored on a defined nano-sized scale with low polydispersity and present a high functionality which allows the conjugation of multiple units of drugs, targeting moieties, solubilizing agents as well as agents which could reduce potential toxicity. The loading ability, water solubility, biodistribution properties, and therapeutic efficacy can be easily tuned by varying structural properties such as the core, branching units, surface groups, or the size of the dendritic core.

In recent years many studies were published using dendritic polymers in gene and drug delivery, magnetic resonance, and fluorescence imaging. Several examples of dendritic molecules have been introduced for biomedical applications such as polyamidoamine,[10] polylysine,[13] polyester,[14,15] polyglycerol,[16–19] and triazines dendrimers.[20]

Chemical conjugation to a dendritic scaffold, thereby exploiting the high multivalency, allows for the covalent attachment of different kinds of active molecules (imaging agents, drugs, targeting

* Corresponding authors. Tel.: +49 761 2062930; fax: +49 761 2062905 (F.K.), tel.: +49 30 838 52633; fax: +49 30 838 53357 (R.H.).
E-mail addresses: felix@tumorbio.unifreiburg.de (F. Kratz), haag@chemie.fu-berlin.de (R. Haag).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.05.058

moieties, or biocompatible molecules) in a controlled ratio. The loading as well as the release can be tuned by incorporating cleavable bonds which can be degraded under the specific conditions present at the site of action (e.g., acidic pH, over-expression of specific enzymes or reductive media).

The covalent attachment of drug molecules to polymers is a promising route for better controlling the loading and release of active molecules as compared to physical encapsulation. Therefore, there is a need for systems where the conjugation and the release of drugs are strictly controlled using biodegradable dendritic scaffolds.[21]

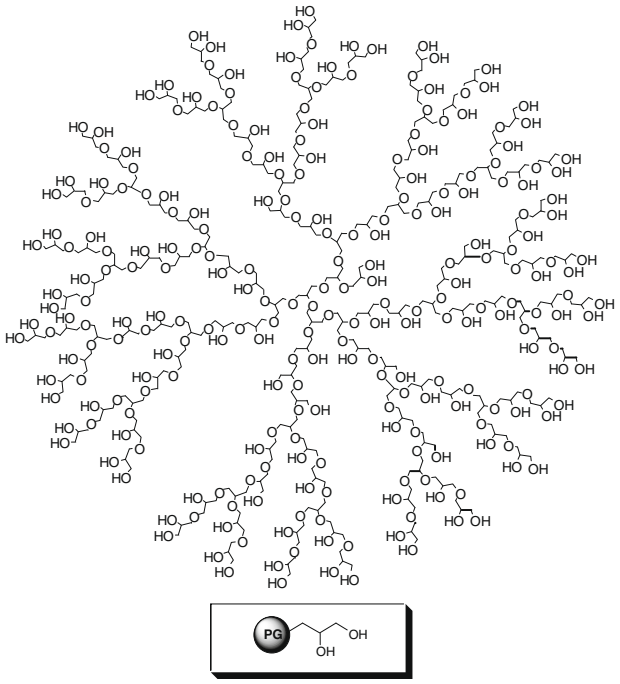


Figure 1. Schematic representation of hyperbranched polyglycerol (PG).

Here we present the synthesis of a scaffold derived from hyperbranched polyglycerol (PG, Fig. 1) and its subsequent conjugation with enzymatically cleavable prodrugs of doxorubicin and methotrexate (Fig. 2),[22,23] and report on the release behavior as well as the cytotoxicity of the new drug polymer conjugates against two human tumor cell lines.

The increasing development of maleimide-bearing prodrugs and diagnostic dyes[23,24] instigated us to synthesize thiolated nanocarriers with tuneable properties such as molecular weight, solubility, or targeting potential by selective functionalization of polyglycerol hydroxyl groups.[25] Therefore, we developed a strategy to synthesize thiolated hyperbranched polyglycerols that can be used as a general, flexible method to couple diagnostic or therapeutic agents under physiological conditions.

The synthetic protocol consisted of four steps. The first three steps, shown in Scheme 1, were carried out as reported earlier for the synthesis of polyglycerolamine with an average molecular weight of 20 kDa and 20% of total hydroxyl groups functionalized to amine groups.[26] For the synthesis of the thiolated derivatives, three different pathways were studied using 3-(tritylthio)propionic acid (A), 2-iminothiolane (B), or acetyl-thiopropionic acid (C). In each case the optimal conditions for synthesis and purification were studied as a function of the reaction time, solvent, stoichiometry and purification method using UV–vis and ^{1}H NMR spectroscopy (see Supplementary data).

Among all the thiolation processes studied, the 2-iminothiolane pathway (B) was the most reproducible for the in situ Michael reaction with maleimide derivatives as the following step. The thiol formation over time was confirmed by ^{1}H NMR and Ellman's test. After 5, 15, 30, 60, 90, 120, 150, 180, 270, 330, and 1220 min samples were taken from the reaction mixture and evaluated. A signal shift from 3.21 to 2.58 ppm in the ^{1}H NMR spectra corresponding to the methylene group in position 2 from 2-iminothiolane as well as the absorbance at 412 nm in UV–vis after incubation with 5,5′-dithio-bis(2-nitrobenzoic acid) was used for monitoring the degree of thiolation. A maximum in the degree of thiolation was reached after 40 min. Standardization of this step

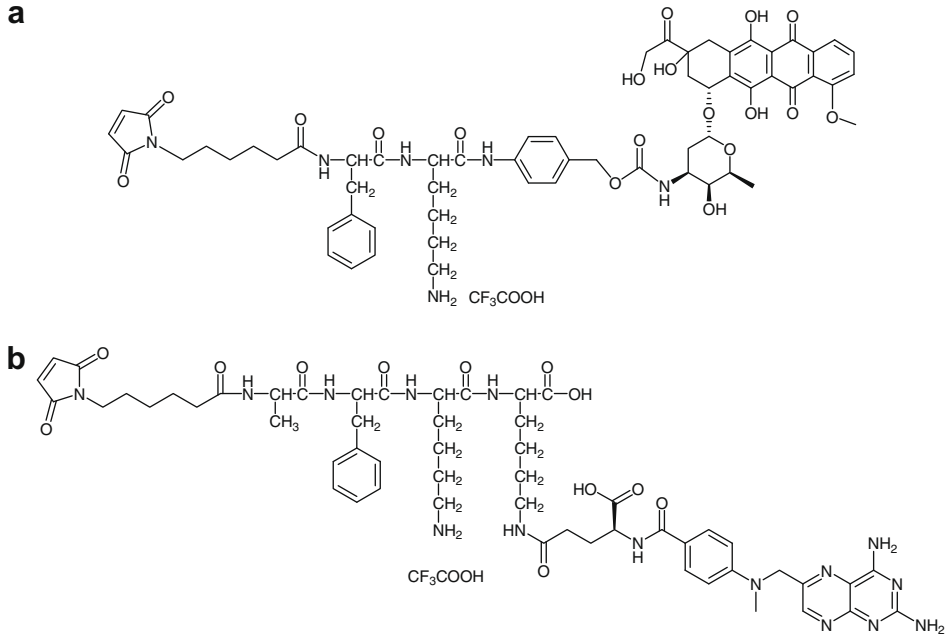
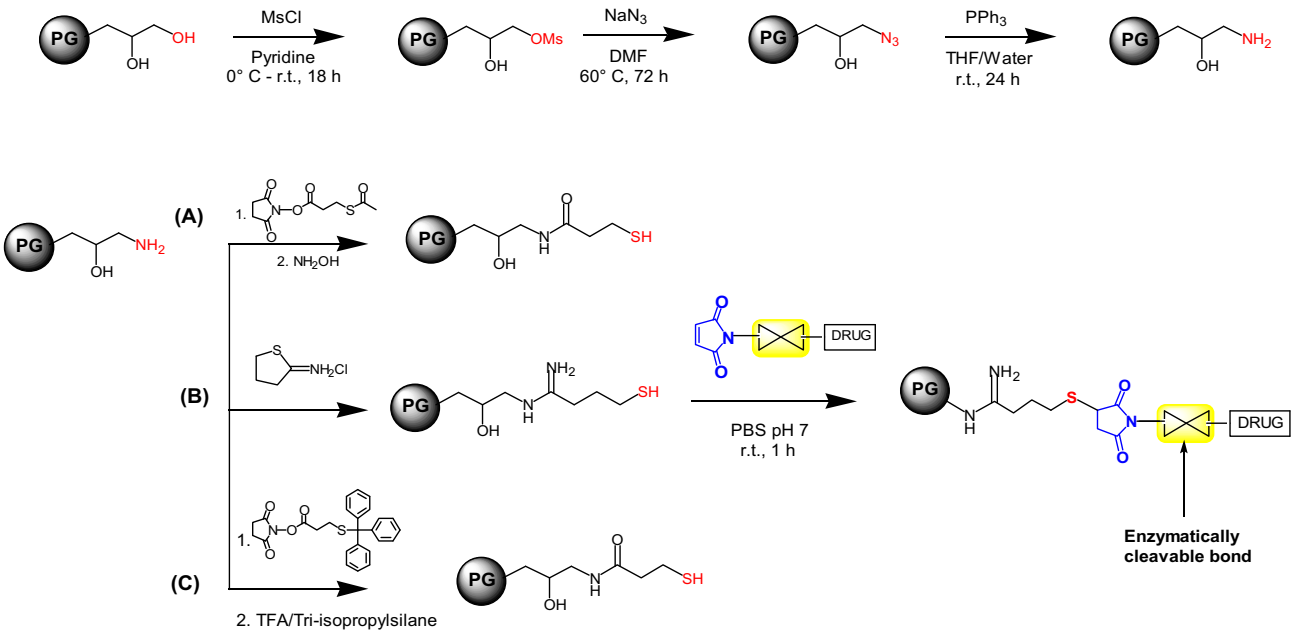


Figure 2. Chemical structures of (a) EMC-Phe-Lys-DOXO, and (b) EMC-D-Ala-Phe-Lys-Lys-MTX (EMC = ε-maleimidocaproic acid).



Scheme 1. Studied pathways for synthesis of thiolated polyglycerol and prodrug coupling.

allowed a convenient control over the amount of thiols generated for further drug conjugation that was performed as a one pot reaction in aqueous systems.

For conjugation, we used a maleimide-bearing prodrug of doxorubicin or methotrexate which incorporate either a self-immolative para-aminobenzyloxycarbonyl (PABC) spacer coupled to the dipeptide Phe-Lys or the tripeptide D-Ala-Phe-Lys as the protease substrate (see Fig. 2). Both prodrugs have been shown to selectively bind to the cysteine-34 position of human serum albumin, they are cleaved by cathepsin B, an enzyme overexpressed by several solid tumors,[27] to release doxorubicin or a methotrexate lysine derivative and they exhibit superior antitumor efficacy in vivo over the free drug.[22,23]

The conjugation between thiolated polyglycerol and the prodrugs was achieved through selective Michael addition between the maleimide group of the prodrugs and the sulfhydryl groups from thiolated polyglycerol in PBS of pH 7. The thiol group adds to the double bound of the maleimide group in a fast and selective reaction at room temperature forming a stable thioether bond. Highly water soluble conjugates, PG-Phe-Lys-DOXO and PG-D-Ala-Phe-Lys-Lys-MTX, were obtained with this protocol with payloads of 45% and 23% wt/wt for doxorubicin and methotrexate, respectively.[30]

Subsequently, drug release of the conjugates was studied in the presence of cathepsin B using size exclusion HPLC.[31] An effective cleavage of PG-Phe-Lys-DOXO and PG-D-Ala-Phe-Lys-Lys-MTX and release of doxorubicin and methotrexate-lysine in presence of the enzyme was observed. As an example, the chromatogrammes for the time-dependent release of doxorubicin are shown in Figure 3 that demonstrate that doxorubicin is liberated over time.

The antiproliferative activity of doxorubicin, methotrexate and the PG drug conjugates was assessed against two human tumor cell lines, AsPC1 LN (pancreatic carcinoma) and MDA-MB-231 LN (mamma carcinoma), which were both transfected with the luciferase gene using a luciferase assay (see Table 1).[32] Whereas in the case of doxorubicin, the IC_{50} values for the PG drug conjugates were lower than for the free drugs, the methotrexate conjugates appeared more active than the free drug. MDA-MB231 cells have been shown to be deficient in methotrexate uptake.[28]

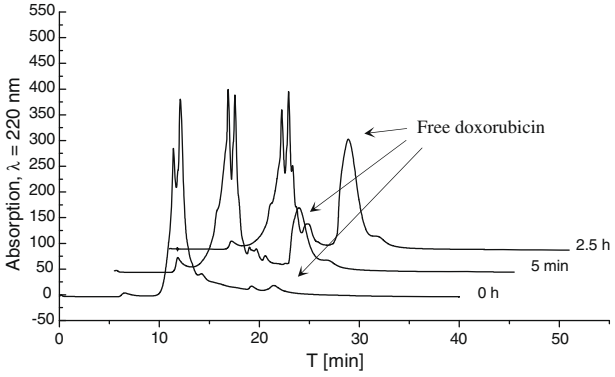


Figure 3. Size-exclusion chromatogrammes of an incubation study of PG-Phe-Lys-DOXO at pH 5.0 and 37 °C with cathepsin B. The cleavage product doxorubicin elutes at ~20 min.

An alternate uptake of the drug in its macromolecular form, followed by intracellular cleavage, might explain the improved activity observed. In any case, however, the conjugates are active in the low micromolar range that is relevant for further preclinical studies of anticancer prodrugs.

Table 1
IC_{50} values of doxorubicin, methotrexate, PG-Phe-Lys-DOXO, and PG-D-Ala-Phe-Lys-Lys-MTX

Compounds	MDA-MB 231 IC_{50} [μM ±SD]	AsPC1 IC_{50} [μM ±SD]
Doxorubicin	0.13 ± 0.06	0.26 ± 0.09
PG-Phe-Lys-DOXO	1.10 ± 0.4	2.4 ± 0.6
Methotrexate	>100 μM	>100 μM
PG-D-Ala-Phe-Lys-Lys-MTX	13.30 ± 0.9	8.9 ± 1.8

For IC_{50} measurements, 0.2×10^4 cells were plated per well in a 96-well plate and serial dilutions of the drugs were added in triplicates. After 72 h cells were lysed in 100 μL of luciferase assay buffer, and 10 μL of the lysate were assayed for luciferase activity. Methotrexate treatment did not reduce the cell proliferation rate below 50% during the experiment.

In summary, we have developed a new macromolecular nanocarrier from a dendritic polyglycerol scaffold. Optimal conditions of synthesis and purification were studied for three different intermediates pathways. Selective conjugation with maleimide-bearing prodrugs as well as drug release in the presence of cathepsin B has been successfully demonstrated. Cytotoxicity of the conjugates against human tumor cell lines showed that the activity of the drugs was primarily retained which encouraged us to study the physical properties and use these systems for further in vivo studies.

Acknowledgments

We thank the Ministry of Science for their continuing support of this work for a NanoFuture award for R. Haag and F. Kratz (BMBF 03X5501).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.058.

References and notes

1. Haag, R.; Kratz, F. *Angew. Chem., Int. Ed.* **2006**, *45*, 1198.
2. Duncan, R. *Nat. Rev. Cancer* **2006**, *6*, 688–701.
3. Vicent, M. V.; Dieudonné, L.; Carbajo, R. J.; Pineda-Lucena, A. *Expert Opin. Drug Delivery* **2008**, *5*, 593.
4. Kiick, K. L. *Science* **2007**, *317*, 1182.
5. Chytil, P.; Etrych, T.; Koňák, Č.; Šírová, M.; Mrkvan, T.; Bouček, J.; Říhová, B.; Ulbrich, K. *J. Controlled Release* **2008**, *127*, 121.
6. Kratz, F.; Abu Ajaj, K.; Warnecke, A. *Expert Opin. Invest. Drugs* **2007**, *16*, 1037.
7. Kratz, F. *J. Controlled Release* **2008**, *132*, 171.
8. Hespe, W.; Meier, A. M.; Blankwater, Y. J. *Arzneim.-Forsch.* **1977**, *27*, 1158.
9. Liu, M. J.; Fréchet, J. M. J. *Pharm. Sci. Technol. Today* **1999**, *2*, 393.
10. Lee, C. C.; MacKay, J. A.; Fréchet, J. M. J.; Szoka, F. C. *Nat. Biotechnol.* **2005**, *23*, 1517.
11. Tomalia, D. A.; Reyna, L. A.; Svenson, S. *Biochem. Soc. Trans.* **2007**, *35*, 61.
12. Svenson, S. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 445.
13. Sadler, K.; Tam, J. P. *J. Biotechnol.* **2002**, *90*, 195.
14. Ihre, H.; Padilla de Jesus, O. L.; Szoka, F. C. J.; Fréchet, J. M. J. *Bioconjugate Chem.* **2002**, *13*, 433.
15. Grinstaff, M. W. *Chemistry* **2002**, *8*, 2839.
16. Haag, R.; Sunder, A.; Stumbé, J. F. *J. Am. Chem. Soc.* **2000**, *122*, 2954.
17. Xu, S.; Luo, Y.; Graeser, R.; Warnecke, A.; Kratz, F.; Hauff, P.; Licha, K.; Haag, R. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1030.
18. Frey, H.; Haag, R. *J. Biotechnol.* **2002**, *90*, 257.
19. Boysen, M. M. K.; Elsner, K.; Sperling, O.; Lindhorst, T. K. *Eur. J. Org. Chem.* **2003**, 4376.
20. Neerman, M. F.; Zhang, W.; Parrish, A. R.; Simanek, E. E. *Int. J. Pharm.* **2004**, *281*, 129.
21. Khandare, J. J.; Minko, T. *Prog. Polym. Sci.* **2006**, *31*, 359.
22. Abu Ajaj, K.; Graeser, R.; Fichtner, I.; Kratz, F. *Cancer Chemother. Pharmacol.* **2009**, *64*, 413.
23. Warnecke, A.; Fichtner, I.; Sass, G.; Kratz, F. *Arch. Pharm.* **2007**, *340*, 389.
24. Tyagarajan, K.; Pretzer, E.; Wiktorowicz, J. E. *Electrophoresis* **2003**, *24*, 2348.
25. Kratz, F.; Müller, I. A.; Ryppa, C.; Warnecke, A. *ChemMedChem* **2008**, *3*, 20.
26. Roller, S.; Zhou, H.; Haag, R. *Mol. Diversity* **2005**, *9*, 305.
27. Jedeszko, C.; Sloane, B. F. *Biol. Chem.* **2004**, *385*, 1017.
28. Worm, J.; Kirkin, A. F.; Dzhandzhugazyan, K. N.; Guldberg, P. *J. Biol. Chem.* **2001**, *276*, 39990.
29. Haag, R.; Mecking, S.; Türk, H. Patent Application DE10211664A1, 2002.
30. Note 1. *General procedure for prodrug coupling*: Polyglycerol (M_n = 20,000 g/mol, PD = 2.0) was prepared according to published procedures.[29] Polyglycerol amine (DF = 20%) was prepared as previously described.[21] The conjugation reactions were performed at room temperature with vigorous stirring for 80 min. To two different flasks, containing 10 mL of a solution of polyglycerolamine (10 mg/mL, 2.7 mmol NH_2-groups) in sterile 5 mM EDTA, 50 mM PBS solution pH 7.0 were added 4.5 mL of a solution of 2-iminothiolane (2 mg/mL in the same solvent system, 1.2 equiv). After 20 min, prodrug solutions were added (3 mg/mL in 5% D-(+)-glucose pH 3.0 for EMC-Phe-Lys-Doxo and 3.7 mg/mL in PBS solution for EMC-D-Ala-Phe-Lys-Lys-MTX). The total amounts of each prodrug were added in three repeated aliquots until the first evidence of precipitation was apparent, waiting ten minutes for addition of each aliquot. After 1 h of reaction, the solutions were concentrated to 5 mL using Centriprep YM10 (twice at 4000 U/min for 30 min). Polyglycerol-drug conjugates were purified by gel-filtration through a Sephadex G-25 column (Amersham) with PBS solution (pH 7) yielding approximately 10–15 mL of a red and yellow solution, respectively. A second concentration with Centriprep was made and finally the conjugates were lyophilized to yield a red (for doxorubicin conjugate) or yellow (for methotrexate conjugate) crystalline powder.Conjugate formation was studied by chromatography on reverse phase TLC, by appearance of a faster band in sephadex column, and by SEC-HPLC. Absence of physical encapsulation was investigated by performing the same coupling procedure with polyglycerolamine alone. The drug concentration of the conjugates was determined photometrically at 495 nm (ε_{495} = 10645 M^{-1} cm^{-1}) for the doxorubicin conjugate and at 370 nm (ε_{370} = 7420 M^{-1} cm^{-1}) for methotrexate conjugate, after reconstitution of the lyophilized conjugates in PBS solution pH 5.8.
31. Note 2. *Cleavage studies of dPG drug conjugates with cathepsin B*: Enzymatically active cathepsin B was purchased from Calbiochem (Bad Soden, FRG). Stock solution of the dPG drug conjugates (3000 μM) were mixed with cathepsin B (71.7 μg/mL, 110 mU) in 20 mM sodium acetate buffer pH 5.0, containing L-cysteine (8 mM). The samples were incubated at 37 °C and were collected over 16 h and analyzed by HPLC: column: BioSil SEC 250 from Biorad, mobile phase: 4 mM sodium phosphate, 0.15 M NaCl, 10% acetonitrile (pH 7.4), flow: 1.0 mL/min; Kontron 422 pump and a Kontron 430 detector (at 495 and 220 nm). For peak integration Geminyx software (version 1.91 by Goebel Instrumentelle Analytik) was used. Control studies were carried out without cathepsin B addition and no release was observed after 16 h of incubation.
32. Note 3. *In vitro cellular experiments*: Cytotoxicity with the dPG drug conjugates and free drugs were performed with two human tumor cell lines: AsPC1 LN (pancreatic carcinoma) and MDA-MB-231 LN (mamma carcinoma) both transduced with the luciferase gene. Cells were grown as monolayer cultures in cell culture flasks in DMEM Glutamax I culture medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 10% carbon dioxide at 37 °C. Media were routinely changed every 3 days. For subculture or experiments, cells growing as monolayer cultures were released from the tissue flasks by treatment with 0.05% trypsine/EDTA. For the experiments, cells were used during the logarithmic growth phase.For the cytotoxicity assay, cells were plated at 200,000 cells/well into a 96-well plate. Stock solutions of the dPG drug conjugates (3 mM) were diluted with DMEM culture medium to 0.01–10 μM and exposed to the cells for 72 h in triplicate. Cells were then lysed in 90 μL of luciferase assay buffer (25 mM TRIS-phosphate pH 7.8; 2 mM EDTA; 2 mM DTT; 0.1% Triton X-100), and 10 μL of the undiluted, as well as 1/10 and 1/100 dilutions were measured in a Luminometer (BMG Lumistar) using the luciferase substrate from Promega (Promega E4550), according to the manufacturer's instructions. IC_{50} values were determined using GRAPHPAD prism software.