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Research Article

Synthesis and In Vitro Cytotoxic Activity on Human Anaplastic Thyroid Cancer Cells of Lipoamino Acid Conjugates of Gemcitabine

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Lipophilic derivatives of the antitumor drug gemcitabine (GEM) with the potential for improving drug loading in lipid-based colloidal carriers, like liposomes or lipid nanoparticles, are described. GEM free base was conjugated to lipoamino acids bearing an alkyl side chain of different length, by either a carbodiimide-assisted or an ethylchloroformiate-assisted coupling reaction, to obtain N^4-acyl GEM derivatives. These compounds retained the same in vitro cell growth inhibitory activity of the parent drug against two lines of human anaplastic thyroid cancer cells. Stability studies suggested that the observed activity was due mainly to intact derivatives and not to released GEM. Accordingly, these amphiphilic derivatives can be proposed in a further step for the encapsulation in liposomes or lipid nanocarriers, to achieve as a final goal an improvement of the pharmacokinetics and therapeutic activity of GEM. Drug Dev Res 71:294–302, 2010.

Key words: lipophilicity; Aro cells; 8305C cells; anaplastic thyroid carcinoma; HPLC determination

INTRODUCTION

The anticancer drug gemcitabine (2′,2′-difluorodeoxycytidine or 2′,2′-difluorodeoxyribofuranosylcytosine, dFdC) (GEM; Fig. 1) is a synthetic pyrimidine nucleoside analogue of cytarabine. GEM is a prodrug that becomes active upon bioconversion by deoxycytidine kinase to its 5′-diphosphate (dFdCDP) and 5′-triphosphate (dFdCTP) metabolites [Plunkett et al., 1996]. Both dFdCDP and dFdCTP inhibit the processes required for DNA synthesis [Plunkett et al., 1995].

GEM is cytotoxic in vitro against a wide range of cell lines derived from solid tumors [Galmarini et al., 2002] and has been approved by the FDA for the treatment of advanced pancreatic carcinoma and for non-small cell lung cancer. In clinical practice, GEM is

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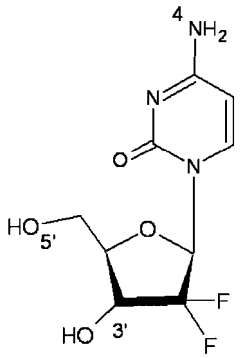

Fig. 1. Structure of gemcitabine, showing position of functional groups.

also useful against breast, head and neck, colon, ovarian, and bladder cancers [Chen et al., 2002; Fuxius et al., 2002; Hertel et al., 1990; Nogue et al., 2002]. GEM is generally well tolerated, with leukopenia and thrombocytopenia the more frequent side effects, and is thus often used in combination with other anticancer drugs.

Anaplastic thyroid carcinoma (ATC) is an aggressive and usually rapidly fatal tumor with a median survival after diagnosis of 6 months [Ain et al., 2000; Are and Shaha, 2006; Haddad et al., 2005]. It accounts for 1–2% of all thyroid carcinomas [Giuffrida and Gharib, 2000; Smallridge et al., 2009]. Current therapy consists of surgery, radiotherapy or radiochemotherapy and chemotherapy. Chemotherapy has evolved from monotherapy with doxorubicin to combination therapy [Ain et al., 2000; Are and Shaha, 2006]. Despite this ATC still has a dismal prognosis.

GEM has shown a potential clinically relevant activity in some ATC cell lines as a single agent [Voigt et al., 2000, 2005]. In a preclinical study, GEM demonstrated marked cytotoxic activity against poorly differentiated human thyroid carcinoma cell lines [Ringel et al., 2000].

After intravenous administration, GEM undergoes a very rapid degradation by plasma deamination [Plunkett et al., 1995]. A potential approach to overcome such limit is to incorporate GEM inside long circulating liposomes [Immordino et al., 2004]. Celano et al. [2004] showed that incorporation of GEM in pegylated neutral liposomes led to an enhancement of the in vitro cytotoxic effect against a human thyroid cancer cell line (ARO) with respect to the free drug, using lower drug concentrations and a shorter cell exposure time (24 h instead of 72 h for free GEM). The same group demonstrated the efficacy of GEM encapsulated in pegylated unilamellar vesicles against Aro cells, as well as against pancreatic cancer cells and multiple myeloma cells [Celia et al., 2008a,b; Cosco et al., 2009].

Because of its polar nature, loading levels of GEM in liposomal bilayers can be limited by this technological approach. A combination of different methods for vesicle preparation has been proposed [Celano et al., 2004]. Moog et al. [2002] described a liposomal gel formulation (GemLip) in which GEM coexists in a liposome-entrapped and free form. This was efficacious in different human cancer models, as well as in murine pancreatic cancer [Bornmann et al., 2008; Graeser et al., 2009; Jantscheff et al., 2009].

Alternatively, converting drugs into more lipophilic labile derivatives or prodrugs can be advantageous in improving loading and in vivo retention time in liposomes [Schwendener and Schott, 2005; Taneja et al., 2000]. The latter strategy has been described by Cattel and coworkers for GEM itself, using N^4-acylamido prodrugs [Brusa et al., 2007; Immordino et al., 2004], which showed antitumor activity in experimental models. In this instance, incorporation in liposomal vesicles achieved the double aim of improving drug incorporation in the liposomes and ensuring chemical protection of the amine group in the 4-position of GEM, i.e., the site of deamination of the drug in plasma. The incorporation of 4(N)-squalenoyl-GEM prodrug in liposomes specially increased the plasma half-life, resulting in a higher level of accumulation in grafted HT-29 and KB 396p tumor cells and antitumor effects in vivo [Brusa et al., 2006]. Squalenoyl prodrugs also increase the potency of GEM after oral administration in leukemia-bearing rats [Reddy et al., 2008].

Different chemical (prodrugs) and technological (colloidal carriers) strategies for delivery and targeting of GEM has been recently reviewed by Reddy and Couvreur [2008].

In recent years, the efficacy of conjugation of antitumor drugs with lipoamino acid residues (LAA) has been evaluated in ours and other groups. LAA are α-amino acids bearing an alkyl side chain in 2-position, whose length and structure can be modified to achieve the required physicochemical properties [Toth, 1994]. Because of the simultaneous presence of an alkyl chain and the polar amino acid head, LAA can impart, to the drugs to which they are conjugated, amphiphilic properties, the so-called "membrane-like character" that ultimately favors their interaction with and penetration through biological membranes and barriers [Toth, 1994; Wong and Toth, 2001]. Linked to alkylating agents like chlorambucil, LAAs increased selectivity toward tumor cells [Wood et al., 1992]. Methotrexate-LAA conjugates showed an enhanced ability in penetrating resistant tumor cells by means of a passive internalization through the cell membrane [Pignatello et al., 1998, 2000, 2001, 2004]. Paclitaxel-LAA

prodrugs have been shown to modify the onset of activity of the drug in vitro against a human ATC cell line (Aro cells), as well as to improve its passive uptake by these tumor cells [Pignatello et al., 2009].

LAAs facilitate the interaction of drugs in a biomembrane model (multilamellar phospholipid liposomes). Their conjugation to a drug molecule in fact, led to amphiphilic compounds that develop more complex interactions with biomembrane components than that seen with simple lipophilic aliphatic moieties [Pignatello et al., 2006]. For GEM, the presence of the sugar moiety in the drug molecule further contributes to the formation of amphipatic conjugates by the addition of one LAA residue. The aim of the present research was to synthesize GEM derivatives using LAA moieties with different lengths of the side alkyl chain (12–16 carbon atoms) and test their intrinsic cytotoxicity against human ATC cell lines.

MATERIALS AND METHODS

^{1}H-NMR spectra were obtained in $CDCl_3$ with a Varian Inova instrument operating at 200 MHz. Chemical shifts are reported in ppm, using TMS as internal standard. Mass spectra were registered with a triple quadrupole instrument (PE Sciex API 3000) operating in SIM mode with positive ion electrospray.

The 2-amino-alkanoic acids were synthesized and Boc-protected before use following a published procedure [Gibbons et al., 1990]. Commercial dry solvents were further dried by distillation according to standard procedures [Perrin, 1980] and storage over 4 Å molecular sieves activated > 24 h at 400°C.

Preparation of GEM Free Base and GEM Hydrochloride

The content of a vial of the commercial medicine Gemzar® (Eli Lilly Italia SpA, Sesto Fiorentino, Italy), containing 1.14 g (3.81 mmol) of GEM hydrochloride, 1 g mannitol, and 62.5 mg sodium acetate, was dissolved in 20 ml of distilled water and was accurately neutralized using 0.1 N and then 0.01 N NaOH (827 pH meter; Metrohm Italiana srl, Origgio, Italy). The solution was lyophilized overnight (Edwards Modulyo, Cinquepascal S.r.l., Trezzano S/N, Milan, Italy) and the resulting white powder placed directly on a flash chromatographic column (silica gel 60, 230–400 mesh ASTM; Merck, Darmstadt, Germany). The column was eluted with chloroform/ethanol mixtures (90:10, 85:15, and finally 70:30, v/v), obtaining ~0.84 g of GEM free base. GEM hydrochloride was prepared by dissolving the free base in 0.1 N HCl and freeze-drying the solution [Cavallaro et al., 2006].

General Procedure for the Synthesis of N^4-Amide GEM Derivatives

Method A

A method similar to the literature for the selective N^4-acylation of Ara-C was used [Tokunaga et al., 1988]. In a three-neck round-bottomed flask, a solution of 0.5 mmol of each N-Boc-protected LAA (kept overnight at 40°C on P_2O_5 and under high vacuum) in 2 ml dry THF was added with triethylamine (TEA, 0.5 mmol). The mixture was cooled to −15°C and 95 µl (1 mmol) ethylchloroformiate diluted with 0.5 ml dry THF added dropwise using magnetic stirring. The reaction was kept at the above temperature for approximately 30 min, during which time the mixture became milky. GEM HCl (0.5 mmol) dissolved in 0.5 ml of dry N,N-dimethylformamide (DMF) and 70 µl (0.5 mmol) of TEA were then added and the temperature was gradually increased to 5°C until the reaction was complete (usually 24–48 h). The time course of the reaction was monitored by TLC [dichloromethane-methanol (DCM-MeOH), 9:1, v/v] performed on silica gel aluminum plates (Merck $F_{254–366}$); the spots were detected by spraying the plate with ninhydrin or an acid–base reactant. The mixture was then evaporated in vacuo and purified by semi-preparative TLC (0.5-mm silica gel plates, Macherey-Nagel GmbH, Düren, Germany), using a 75:25 DCM–MeOH eluent mixture, to yield the N-Boc-protected LAA derivatives. Treatment of the latter with a TFA/DCM 1:1 solution, at room temperature for 30–40 min, gave the final compounds **1–3** with a final mean yield of 69%.

Method B

First, 3′,5′-di-Boc GEM was obtained as described in the literature [Guo and Gallo, 1999]. GEM HCl was dissolved in 1 M aqueous KOH and reacted with a 10-fold excess di-*tert*-butyl dicarbonate (DBDC) in dioxane. After extraction of the reaction products (a mixture of mono- and di-Boc protected GEM), further addition of DBDC and KOH provided high yields of the required 3′,5′-di-Boc protected drug.

The carboxyl group of the Boc-LAA (0.1 mmol) was activated in 2 ml dry DCM (Aldrich) with a slight excess of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDAC), in the presence of equimolar amounts of 1-hydroxybenzotriazole and TEA, for 2 h in an iced water bath. A solution of 3′,5′-di-Boc GEM (0.1 mmol) in 2 ml dry DCM and 0.5 ml dry DMF was then added and the reaction kept at 0°C for 2 h and then at room temperature for 24 h. The course and completion of the reaction were monitored by TLC under the above described conditions.

The reaction products were isolated by semi-preparative TLC as described in the method A. Treatment with TFA/DCM, as described, gave compounds 1–3 with mean yields around 58%.

- *N^4-(2-amino)dodecyl-gemcitabine (**1**).* $C_{21}H_{34}F_2N_4O_5$; ^{1}H-NMR ($CDCl_3$, δ): 8.15 and 7.53 (d, J = 3.8 Hz, 1+1H, pyrimidine), 6.05 (weak t, 1H, furan), 4.23–4.20 (m, 1H, furan =CH–OH), 4.10 (br m, 1H, CH), 3.64–3.58 (m, 2H, furan CH_2–OH), 1.80 (m, 2H, CH_2–CH_3), 1.31–1.25 (m, 16H, CH_2), 0.87 (t, 3H, ω–CH_3). FAB-MS (m/z): 461.6 $[M+1]^+$ (100%).
- *N^4-(2-amino)tetradecyl-gemcitabine (**2**).* $C_{23}H_{38}F_2N_4O_5$; ^{1}H-NMR ($CDCl_3$, δ): 8.02 and 7.34 (d, J = 3.8 Hz, 1+1H, pyrimidine), 6.10 (weak t, 1H, furan), 4.30 (m, 1H, furan =CH–OH), 4.06 (br m, 1H, CH), 3.64–3.49 (m, 2H, furan CH_2–OH), 1.72 (m, 2H, CH_2–CH_3), 1.34–1.25 (m, 20H, CH_2), 0.88 (t, 3H, ω–CH_3). FAB-MS (m/z): 489.6 $[M+1]^+$ (100%).
- *N^4-(2-amino)hexadecyl-gemcitabine (**3**).* $C_{25}H_{42}F_2N_4O_5$; ^{1}H-NMR ($CDCl_3$, δ): 8.21 and 7.40 (d, J = 3.8 Hz, 1+1H, pyrimidine), 6.22 (weak t, 1H, furan), 4.22–4.20 (m, 1H, furan =CH–OH), 3.99 (br m, 1H, CH), 3.86 (m, 2H, furan CH_2–OH), 1.78 (m, 2H, CH_2–CH_3), 1.26–1.19 (m, 24H, CH_2), 0.83 (t, 3H, ω–CH_3). FAB-MS (m/z): 517.5 $[M+1]^+$ (76%); 433 (100%).

HPLC Analysis

The purity of the final compounds was checked by high-performance liquid chromatography (HPLC). Methanol solutions of the compounds (~0.1 mM) were injected into a Waters C18 Symmetry column (5 µm, 4.6 × 150 mm) connected to a Waters C18 Symmetry pre-column and eluted by a 55:45 (v/v) acetonitrile–water mixture, at a flow of 0.8 ml/min. A Varian ProStar instrument was used, equipped with a ProStar 325 UV detector set at 269 nm. Typical elution times under the above conditions were 3.6 min for GEM and 7.5, 9.6, and 11.2 min for compounds **1**, **2**, and **3**, respectively. The diacyl LAA side products were usually detected at higher retention times (around 15–19 min). Each sample was tested in triplicate.

The chemical stability of compounds **1–3** was assessed in triplicate by incubating weighed amounts of each compound (~1 mg) in 20 ml of the cell culture medium used in the biological assays (see below) at 37°C. At predetermined times, samples were gently vortex-mixed and 100-µl aliquots were withdrawn; 900 µl of HPLC-grade methanol was added, and the mixture was centrifuged at 4°C for 30 min at 10,000 rpm using a Beckman (Fullerton, CA) J2-21 centrifuge equipped with a Beckman JA-20.01 fixed-angle rotor. The surnatant was then filtered (0.22 µm nylon membrane; Whatman) and injected in the HPLC system to identify the intact GEM-LAAs and released GEM.

Biological Experiments

Cell lines

The study was performed using two human ATC lines: Aro and 8305C. Cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml penicillin-streptomycin.

MTS assay and cell viability

Cell viability was determined using a colorimetric method, the CellTiter 96 AQ_{ueous} One solution Cell Proliferation Assay (TB245, Promega, Madison, WI), which uses a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent, phenazine ethosulfate (PES), which is combined with MTS to form a stable solution. MTS is chemically reduced by cells into a colored formazan product that, as measured by the absorbance at 490 nm, is directly proportional to the number of living cells in culture.

Cells were seeded into 96-well plates at 5×10^3 cells/200 µl/well and allowed to adhere to the plate overnight. The next day, cells were treated with 1, 5, 10, or 50 µM GEM hydrochloride or an equivalent amount of compounds **1–3**. Dilutions were made using the culture medium from a 10 mM stock solution of each drug in DMSO. After 24, 48, or 72 h of incubation, the culture medium was aspirated and the cells were washed with PBS (pH 7.4); 100 µl of fresh culture medium without drugs and 20 µl of MTS were added to each well, and cells were incubated for additional 3 h. The plates were read on a Microplate Reader (Synergy HT, BIO-TEK). Survival was expressed as the percentage of viable cells in treated sample relative to untreated control cells. Control experiments were made using pure DMSO at concentrations equivalent to the test samples. All experiments were repeated three times in triplicate. Statistical analysis of means was performed by paired Student's *t*-test, using the Microsoft® Office Excel 2003 software.

RESULTS AND DISCUSSION

Chemistry

Selective acylation in the N^4-position of GEM with N-Boc protected LAA was obtained using two alternative methods that have been described in the literature for the same or chemically related drugs. The

first procedure was followed the work of Tokunaga et al. [1988], who described a selective N^4-acylation of Ara-C, a molecule structurally close to GEM. Immordino et al. [2004] used the same method to obtain N^4-acyl derivatives of GEM. In this procedure, a THF solution of the LAA was added of ethylchloroformiate at low temperature (the reaction is exothermic) and in the presence of TEA. After the activation time the free base of GEM was added and the reaction was usually prolonged for 1 or 2 days.

The main risk of this procedure is the side reaction leading to the simultaneous acylation of hydroxyl groups in the GEM molecule [Tokunaga et al., 1988; Wu et al., 2007]. However, in contrast with the report of Immordino et al. [2004], the diacyl-LAA derivatives formed in our reactions were efficaciously removed by semi-preparative TLC.

An alternative synthetic route was the selective Boc-protection of the two (3′ and 5′) hydroxyl groups in the GEM molecule, followed by the acylation of the free N^4-amino group with the Boc-LAA in the presence of a water soluble carbodiimide (EDAC HCl) and the final removal of all the Boc moieties by TFA treatment. The selective protection of the hydroxyl groups was possible using a double treatment of GEM with an excess of DBDC in dioxane, in the presence of 1 M aqueous KOH [Guo and Gallo, 1999].

The structure of the synthesized compounds (Fig. 2) was confirmed by routine analyses. In particular, in the ^{1}H-NMR spectra, the introduction of the LAA moiety was evidenced by the triplet due to the ω-methyl group (~0.85 ppm) and the signals of the aliphatic side chain (~1.6–1.7 and 1.25 ppm). Noteworthy, the pyrimidine protons gave relatively little coupling constants (3.8 Hz); this could be explained with the used solvent (*d*-chloroform), in which the amphipatic GEM-LAA conjugates are likely to form micelles or supra-aggregates.

The hydrolytic stability shown by compounds **1–3** indicated that all of them very slowly release the parent drug in the conditions used for cell culture (Fig. 3). After 72 h, up to 82% of intact conjugates were still found. To confirm that this stability was due to the low degradability of the amide bond between GEM and the LAA residues, a specimen of compound **1** was treated with 1N NaOH. After 4 h at 37°C, an almost complete conversion of GEM-LAAs into the parent drug was registered by HPLC (data not shown).

Biological Results

The cell growth inhibitory activity of GEM and compounds **1–3** was assayed using two human ATC lines, Aro and 8305C. Compounds (1–50 μM) were incubated for 24, 48, or 72 h to assess any difference with respect to free GEM deriving from drug conjugation with the LAA moieties and also in terms of onset and duration of the activity. Controls with pure DMSO confirmed the absence of intrinsic toxicity due to the solvent in both tumor cell cultures.

GEM exhibited time-dependent inhibitory activity at all the tested concentrations against Aro cells. It was effective only after 48 or 72 h of incubation, with a 60% cell growth inhibition measured at the maximum concentration tested (Fig. 4). Compounds **1–3** basically displayed the same profile of activity as GEM against this cell line at the lowest dose (1 μM). However, with increasing concentrations some differences were observed, especially in relation to the incubation time. In detail, compound **1** appeared to be less effective than GEM at longer incubation times. In contrast,

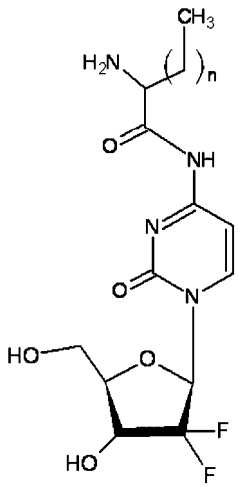

Fig. 2. General structure of GEM-LAA derivatives: 1, $n = 9$; 2, $n = 11$; 3, $n = 13$.

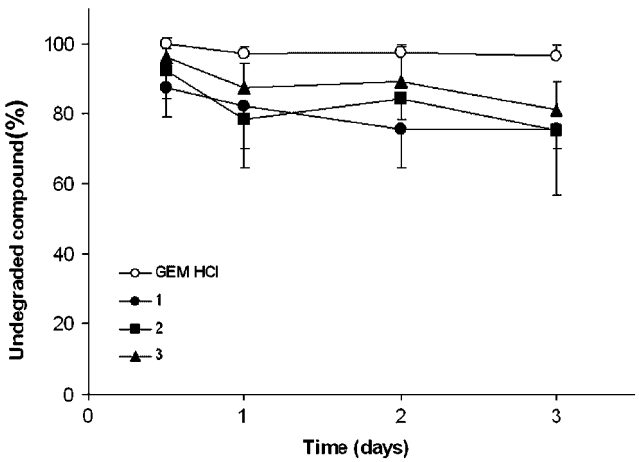

Fig. 3. Hydrolytic stability profiles of compounds 1–3 upon incubation at 37°C with the medium used in the cell culture assays.

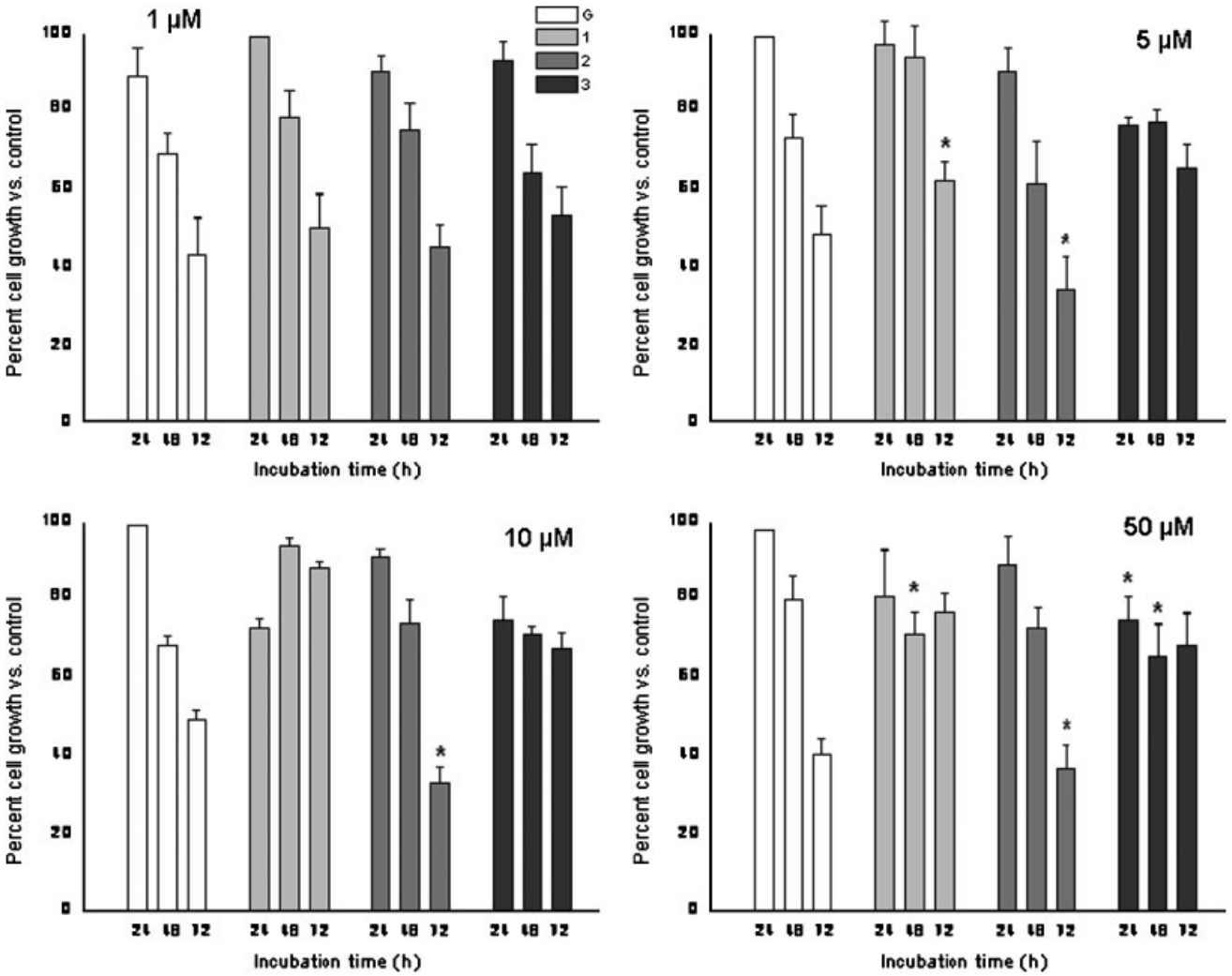

Fig. 4. Time-related cell growth inhibitory activity of GEM and compounds 1–3 at different concentrations against Aro cells. $^{*}P<0.05$ (Student's *t*-test).

compound **2** had an activity very close to that of the parent drug, and was even more active than free GEM at 5 and 10 μM and after 72 h of incubation ($P<0.05$ for both values) (Fig. 4). The higher homologue **3** was slightly less active than GEM against Aro cells, with no significant dependency on the incubation length.

The 8305C cell line was more sensitive to GEM, with a 35–45% growth inhibition already after 24 h of incubation (Fig. 5). The three novel compounds were as active as the parent in inhibiting the growth of this cell line; also in this case, time-dependent inhibitory activity was observed, reaching 80–85% cell growth inhibition after 72 h. The three derivatives shared essentially the same profile of activity. From the stability assay data, the GEM-LAAs appear stable in the applied cell culture conditions suggesting either that these compounds possess intrinsic antitumor activity, close to the parent drug against the tested cell lines, or that they are up taken in a similar way inside cells, where they undergo hydrolysis to free GEM by intracellular enzymes. Further stability studies will aid in elucidating the precise mechanism of action of the newly synthesized compounds.

CONCLUSIONS

A series of amphiphilic derivatives of GEM were obtained by a selective acylation of the drug amine group. The biological evaluation suggested that the conjugation of GEM to the different LAA moieties did not decrease the drug cell growth inhibitory activity in vitro. The improved lipid solubility resulting from the introduction of the LAA residues could however, be helpful in boosting the loading of these derivatives in liposomes or other lipid-based drug carriers. Additional research will explore this potential, with the ultimate

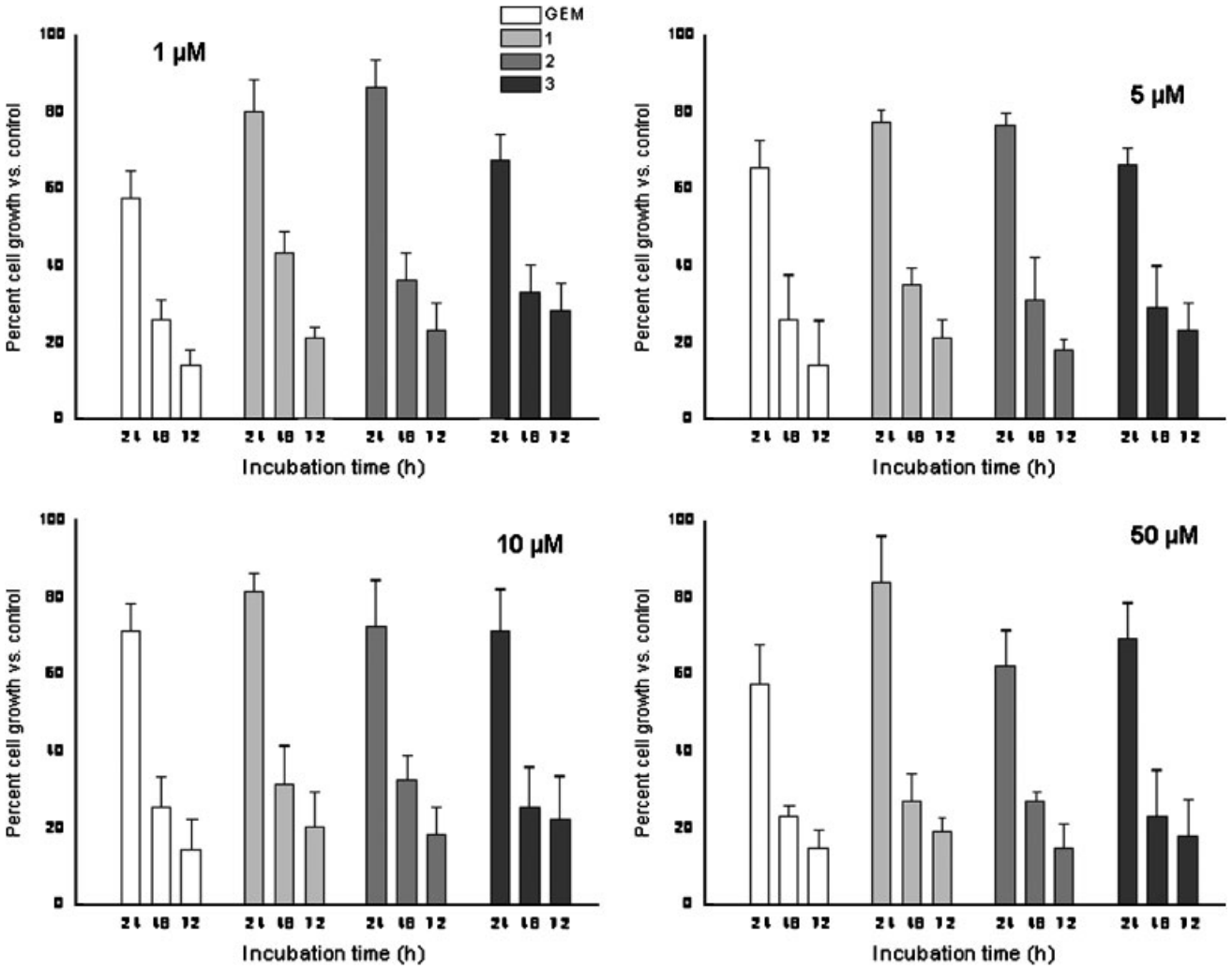

Fig. 5. Time-related cell growth inhibitory activity of GEM and compounds 1–3 at different concentrations against the 8305C cell line.

goal of improving the pharmacokinetic profile, selectivity of action, and possibly systemic tolerability of GEM.

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