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Research paper

Interaction of an amphiphilic squalenoyl prodrug of gemcitabine with cellular membranes

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ABSTRACT

We have designed an amphiphilic prodrug of the anticancer agent gemcitabine (dFdC), by covalent coupling to squalene. This bioconjugate, which self-assembled into nanoparticles (NPs) in water, was previously found to display an impressive anticancer activity both *in vitro* and *in vivo*. The present study aims to investigate the impact of SQdFdC nanoparticles on cellular membranes. MTT assays showed that, in the nanomolar range, squalenoyl gemcitabine (SQdFdC) was slightly less active than dFdC on a panel of human cancer cell lines, *in vitro*. However, above 10 μmol L^{-1} SQdFdC was considerably more cytotoxic than dFdC. Contrarily to its parent drug, SQdFdC also induced cell lysis in a few hours, as evidenced by LDH release assays. Erythrocytes were used as an experimental model insensitive to the antimetabolic activity of dFdC to further investigate the putative membrane-related cytotoxic activity of SQdFdC. The bioconjugate also induced hemolysis in a time- and dose-dependent fashion, unlike squalene or dFdC, which clearly proved that SQdFdC could permeabilize cellular membranes. Structural X-ray diffraction and calorimetry studies were conducted in order to elucidate the mechanism accounting for these observations. They confirmed that SQdFdC could be transferred from NPs to phospholipid bilayers and that the insertion of the prodrug within model membranes resulted in the formation of nonlamellar structures, which are known to promote membrane leakage. As a whole, our results suggested that due to its amphiphilic nature, the cell uptake of SQdFdC resulted in its insertion into cellular membranes, which could lead to the formation of nonlamellar structures and to membrane permeation. Whether this mechanism could be the source of toxicity *in vivo*, however, remains to be established, since preclinical studies have clearly proven that squalenoyl gemcitabine displayed a good toxicity profile.

1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a cytidine analog currently used in clinic against a number of solid tumors. Over the past 15 years, it has been approved for the treatment of locally advanced or metastatic non-small-cell lung cancer gemcitabine plus cisplatin versus cisplatin alone in patients [1] and pancreatic cancer [2], invasive bladder cancer [3] and in second-line against metastatic breast cancer [4]. Phase II clinical trials of this anticancer agent have also been undertaken against cervix [5,6] and colorectal malignancies [7]. The drug exerts its cytostatic and cytotoxic activities mainly through the incorporation of its triphosphate metabolite into elongating DNA, which leads to chain termination, cell cycle blockage and eventually apoptosis [8]. The uptake of dFdC via membrane transporters [9] and its phosphorylation by cytoplasmic kinases are critical prerequisites of this process, and their impairment may lead to the induction of resistance to the chemotherapy [10]. dFdC also undergoes a rapid deamination into its inactive uracil derivative in the bloodstream, which severely limits its bioavailability [11].

We have introduced the concept of "squalenoylation" to improve the efficacy of gemcitabine and to circumvent some of its above-mentioned shortcomings. Squalene (SQ), a natural lipidic precursor of the biosynthesis of cholesterol, was coupled to the amino group of the cytosine nucleus of dFdC. When introduced in water, the resulting bioconjugate spontaneously self-assembled

Abbreviations: dFdC, gemcitabine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; LDH, lactate dehydrogenase; LISB, low ionic strength buffer; MLV, multilamellar vesicles; NP, nanoparticle; SAXS/WAXS, small/wide angle X-ray scattering; SQ, squalene; SQdFdC, squalenoyl gemcitabine.

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into nanoparticles (NPs) [12], which were found dramatically more active than dFdC against murine leukemia models, *in vivo* [13]. Mechanistic studies have proven that squalenoyl gemcitabine (SQdFdC) was not a substrate of the membrane transporters of dFdC [14] but that it rather entered cells by an albumin-enhanced passive diffusion [15]. Structural studies have underlined that the amphiphilic SQdFdC molecules strongly interacted with phospholipids [16], and we have recently reported that cellular membranes constituted the main intracellular reservoir of the prodrug [17].

Numerous authors have indicated that other amphiphilic drugs could interact with cellular membranes [18], which could lead to toxic side effects [19–21]. We, therefore, investigated whether the interaction of squalenoyl gemcitabine with cellular membranes could result in cytotoxic effects caused by their disruption. The cytostatic and cytotoxic activities of SQ, dFdC, and SQdFdC were compared on a panel of human cancer cell lines, *in vitro*. The cytolytic effects of SQdFdC were clearly evidenced, and their dependency with time and drug concentration was assessed on both cancer cell lines and erythrocytes. In addition, differential scanning calorimetry was performed and X-ray diffraction measurements were taken, demonstrating the ability of SQdFdC to be transferred from NPs to DPPC model membranes, which leads to the formation of nonlamellar structures responsible for the enhanced membrane permeability and cell lysis.

2. Materials and methods

2.1. Chemicals and drugs

Ethanol was purchased from Carlo Erba (Italy), all cell culture media from Lonza (France), and other chemicals, including Squalene, from Sigma–Aldrich (France). Ultrapure MilliQ® water was used (Millipore, France). Gemcitabine hydrochloride (dFdC) was obtained from Sequoia Research Products Ltd. (UK). SQdFdC was synthesized, purified, and characterized as previously reported [12]. 1,2-Dipalmitoyl-sn-3-phosphatidylcholine (DPPC) (molecular weight of 733.56, purity 99%) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and used without further purification.

2.2. Preparation of SQdFdC NPs and control SQ dispersions

Two milligrams of SQdFdC was dissolved in 1 mL ethanol and added dropwise under vigorous magnetic stirring to 2 mL of MilliQ® water to generate nanoparticles (NPs). Two milligrams of SQ was dispersed similarly, in the presence of 1% (w/v) Pluronic F68, to stabilize the SQ emulsion. Ethanol was evaporated on a Rotavapor® (Büchi) to reach a final volume of 2 mL. The resulting nano-objects were characterized by quasi-elastic light scattering on a Zetasizer Nano ZS (Malvern) in terms of average diameter (Z-avg) and polydispersity index (Pdi). Typical characteristics of the objects were the following: SQdFdC NPs, Z-avg = 104 ± 1 nm; Pdi = 0.084 ± 0.012 and SQ dispersion, Z-avg = 304 ± 2 nm; Pdi = 0261 ± 0.001. The dispersions were diluted in culture media and added onto cells within 1 h after their preparation.

2.3. Cell lines and culture conditions

The human cell lines MCF-7 and MDA-MB-231 (breast adenocarcinoma), HT-29 (colorectal adenocarcinoma), and KB-3-1 (cervix carcinoma) were grown in Dulbecco's Modified Eagle Medium (DMEM) Hi-Glucose GlutaMAX, in the presence of fetal calf serum (FCS, 10% (v/v)). The human leukemia cell line CCRF CEM was cultured in RPMI 1640/GlutaMAX with 10% (v/v) FCS. Cell culture media were supplemented with penicillin (50 U mL^{-1}) and streptomycin (50 μg mL^{-1}). Cells were grown in a 5% CO_2 humidified atmosphere at 37 °C.

2.4. MTT assay

Cytostatic and cytotoxic effects were monitored thanks to the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) test. Cells were seeded in 96-wells plates, and drug-containing media were added to the exponentially growing cells the day after. After a 48 h exposure to the drugs, each well received MTT (final concentration 0.5 mg mL^{-1}). Two hours later, the plates were centrifuged and the supernatants replaced by DMSO to dissolve the formazan crystals. Absorbance at 570 nm, which is proportional to the number of living cells, was measured on a Metertech Σ 960 microplate reader (Fisher Bioblock). Viability data normalized to exponentially growing untreated control cells were fit either to a Hill function with an additive constant (dFdC) or to a sum of two Hill functions (SQdFdC), by nonlinear least squares on Excel® software (Microsoft) thanks to its built-in "Solver" algorithm.

2.5. LDH release assay

The membrane disruption of human cancer cells was investigated using the lactate dehydrogenase (LDH, a stable cytoplasmic enzyme) release assay thanks to a commercial colorimetric kit (Cytotox 96 nonradioactive assay, Promega, France), according to manufacturer's instructions. Briefly, the supernatant of cells seeded in 96-well plates was replaced by fresh media containing 200 μmol L^{-1} of either dFdC or SQdFdC NPs. After various incubation times at 37 °C, the supernatants were homogenized, plates were centrifuged, and samples were taken for LDH measurement. The cell lysis was calculated relatively to untreated control cells and to cells entirely lysed by 1% (v/v) Triton X100.

2.6. Hemolysis experiments

These experiments were carried out in a cell-compatible low ionic strength buffer (LISB) in order to maintain the colloidal stability of the NPs in a protein-free environment [15]. Control dynamic light scattering measurements carried out either in MilliQ® water, LISB- or FCS-containing culture medium have shown that the colloidal stability of the NPs was similar in all cases. LISB composition was as follows: 250 mmol L^{-1} saccharose, 10 mmol L^{-1} glucose, 15 mmol L^{-1} HEPES buffer, 90 μmol L^{-1} Ca^{2+} and 50 μmol L^{-1} Mg^{2+}, pH set to 7.4. Healthy adult female CD2F1/NCrl mice were anaesthetized by intraperitoneal injection of a ketamine/xylazine solution (resp. 33 mg kg^{-1} and 7 mg kg^{-1}), and their blood was sampled by cardiac puncture, in compliance with the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J No. L358, 18 December 1986). The erythrocytes were separated from serum by centrifugation, washed twice in 10 volumes of LISB, and dispersed in drug-containing LISB according to an hematocrite (erythrocyte volume fraction) of 0.1%, 0.2% or 0.28%. Cell suspensions were incubated at 37 °C and harvested each hour for up to 6 h. The optical density of the media was then assessed at a wavelength of 450 nm on a microplate reader, after removing the cellular bodies by centrifugation. Hemolysis percentage was calculated relatively to the absorbance obtained for erythrocytes in the presence of 1% (v/v) Triton X100 (100% lysis). The order of magnitude of the amount of lipids contained in the hemolysis setup was estimated on the basis of published data [22–26], for comparison to the SQdFdC/DPPC ratio used in DSC-SAXS/WAXS studies.