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Short communication

Regioselective chemical and rapid enzymatic synthesis of a novel redox – Antiproliferative molecular hybrid

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ABSTRACT

Recent science evidenced the interlinkage of oxidative stress and cancer. Due to the inherent complexity of cancer and its accompanying effect of oxidative stress, novel molecules, containing combinatorial functionalities should be targeted. Herein, we synthesized gemcitabine-5′-*O*-lipoate derived from a regioselective coupling of the chemotherapeutic drug gemcitabine (GEM), a first-line agent for cancer therapy and α-lipoic acid (LA), a potent antioxidant. gemcitabine-5′-*O*-lipoate was obtained in 4 chemical steps. To avoid the tedious and laborious chemical steps we also utilized biocatalysis using immobilized *Candida antarctica* lipase B (CALB), and the optimum conditions for the regioselective and one-pot synthesis of gemcitabine-5′-*O*-lipoate were established by exploiting different solvents (organic solvents and ionic liquids) and enzyme immobilization (acrylic resin and carbon nanotubes). Cytotoxic activity of co-administrating GEM and LA was proven to be synergistic against non-small cell lung cancer cells whereas antagonistic for bladder cancer cells. In contrast, the gemcitabine-5′-*O*-lipoate hybrid was found to be superior to the parent compounds against both non-small cell lung cancer and bladder cancer cells as also was found to preserve the redox activity of the parent compound LA. The regioselective biotransformation mediated synthesis of the anticancer-antioxidant hybrid illustrates the capacity of biocatalysis to act as an asset in molecular hybridization techniques.

1. Introduction

The imbalance between the production of free radicals and reactive oxygen species (ROS) leads to oxidative stress, which affects various signaling pathways in many diseases including cancer. The impact of ROS in cancer includes gene mutations, structural changes in DNA as well as abnormal gene expression, modifications of second-messenger system and blockage of cell–cell communication, depending on the stage of cancer [1]. Since cancer cells are vulnerable to agents that further modulate redox sensitive agents, pro-oxidants emerge as an exciting direction to target tumor cells [2]. Although most studies had focused on the antioxidant activity of bioactive compounds, their pro-oxidant properties, that could mediate specific toxicity against cancer cells due to the low level of antioxidant defenses found in tumors, has been largely omitted [2]. Along these lines there is currently a large array of ongoing clinical trials aiming to exploit novel redox drugs in cancer patients [3]. In the quest to optimize the biological activities of drugs for multifactorial diseases, the hybrid approach, i.e. to covalently link two distinct potent entities into one molecule, is a promising area and constantly gains ground [4–9]. As follows, the formulation of a molecular hybrid containing in the same scaffold an anticancer component and an antioxidant/pro-oxidant part, could confer complementary and enhancement biological activity. Thus, we

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exploited the conjunction of gemcitabine (GEM) (**1**) and α-lipoic acid (LA) (**4**) in order to produce a hybrid molecule bearing anticancer and antioxidant/pro-oxidant activity. LA (**4**) is a natural occurring dithiol compound, which is well known as a powerful micronutrient with antioxidant [10,11] and pro-oxidant properties [12–14] (Fig. 1). GEM (**1**) (2′,2′difluorodeoxycytidine, dFdC, Fig. 1) is a synthetic deoxycytidine analog, with potent anti-tumor activity in various human cancers, such as pancreatic cancer, bladder cancer, breast cancer and non-small cell lung cancer [15,16], by promoting reduction of neoplastic cell proliferation and apoptosis [17]. It acts as a pro-drug and once it is transported into the cell it is converted to its active form, gemcitabine triphosphate (dFdCTP), through phosphorylation in its 5′-OH by deoxycytidine kinase. dFdCTP is then incorporated to DNA and causes cell death through inhibition of processes required for DNA synthesis [18]. However, its non-specific toxicity, brief plasma half-life and chemotherapeutic-resistance, acquired i.e. through reduction in the expression of nucleoside transporters responsible for its intracellular uptake, have restricted its utility in clinical oncology. A strategy to reduce its non-specific toxicity as also to surmount deficits in its intracellular uptake in cancer cells could be through its conjugation with LA (**4**). GEM (**1**) conjugation to LA (**1**) should be conducted in a regioselective way and the ideal position will be its 5′-OH, which participates to the phosphorylation leading to its DNA incorporation and cell death [6,19,20]. To achieve this synthesis we followed both classical chemical synthesis as also biotransformation-mediated synthesis in an effort to minimize the laborious multistep reactions required to the synthesis of such molecular hybrids. The resulted hybrid molecule, gemcitabine-5′-*O*-lipoate (**6**), was evaluated for its cytotoxic efficacy against two cancer and one normal human cell lines, by measuring cell growth inhibition. In vitro chemosensitivity of the tested cell lines was also evaluated for the isolated chemotherapeutic drug (GEM) (1) and (LA) (**4**) treating cells either with each compound alone, or in simultaneous and sequential mode of exposure. The combined drug interaction was assessed with the median-effect analysis and the combination index (CI). gemcitabine-5′-*O*-lipoate (**6**) was also exploited for its redox potential in cells.

2. Results and discussion

2.1. Chemical synthesis of the molecular hybrid gemcitabine-5′-*O*-lipoate (*6*)

The chemical synthetic method for gemcitabine-5′-*O*-lipoate (**6**) is depicted in Fig. 2A. LA (**4**) can be coupled to (**1**) at 4-NH_2, 3′-OH, and 5′-OH positions. Development of the gemcitabine-5′-*O*-lipoate conjugate requires selective protection at 4-NH_2 and 3′-OH of GEM (**1**) [6,21] prior to LA (**4**) coupling. Transformation of (**1**) into compound (**6**) was achieved via a four-step synthetic process. In order to regioselectively couple LA (**4**) to the 5′-OH position of (**1**), we followed a selective protection/deprotection scheme for the 4-NH_2 and 3′-OH positions. The *tert*-butoxycarbonyl (Boc) group was used as selective protecting group and the synthetic paths previously reported were followed [6,21]. Briefly, in the first step, the 3′-OH of (**1**) was protected with Boc using di-*tert*-butyl dicarbonate (DBDC) to give product (**2**). Consecutively, (**2**) was protected at the 4-NH_2 with DBDC to give product (**3**). (**3**) was then coupled at its 5′-OH with LA (**4**) with *N,N*′-Dicyclohexylcarbodiimide (DCC) to obtain product (**5**). Finally, TFA mediated Boc deprotection gave the desired gemcitabine-5′-*O*-lipoate product (**6**). The reaction yields for the four steps were found to be 94%, 60%, 26% and ~80%, respectively.

2.2. In silico studies to evaluate the potential of CALB to regioselectively biotransform gemcitabine (*1*) and α-lipoic acid (*4*) into a molecular hybrid

The chemical synthesis of gemcitabine-5′-*O*-lipoate (**6**) required 4 laborious and time consuming steps. To avoid the tedious protection/deprotection steps followed during the classical synthetic process, illustrated above, we exploited the possibility to achieve the desired molecular hybrid in one-pot after regioselective biotransformation-mediated synthesis. *Candida antarctica* lipase B (CALB) is a broadly used biocatalyst that confers regio-selectivity towards the more nucleophilic primary hydroxyl groups of the substrate molecule [22,23]. Initially, we explored *in silico* the potential favorable accommodation of the gemcitabine-5′-*O*-lipoate (**6**) in the catalytic site of CALB. The structure of gemcitabine-5′-*O*-lipoate (**6**) was initially optimized by conformational search using the Monte Carlo method with the MMFF94 molecular mechanics model. Geometry optimization was accomplished via quantum-chemical calculations by utilizing *ab initio* Hartree − Fock method with 6-31G* basis set (Fig. S2). Docking calculations of gemcitabine-5′-*O*-lipoate (**6**) to CALB employed full ligand flexibility and partial protein flexibility focused at the ligand binding site. Molecular docking calculations resulted in very favorable energies for gemcitabine-5′-*O*-lipoate (**6**) docking (−36.13 kcal/mol). Molecular docking of gemcitabine-5′-*O*-lipoate (**6**), GEM (**1**), LA (**4**) and co-crystallized inhibitor HEE are depicted in Fig. 3. All molecules are shown to be stabilized inside the protein's binding pocket and the same place where its co-crystalized inhibitor HEE is bound. Gemcitabine-5′-*O*-lipoate (**6**) is found to be positioned inside the binding cavity with it's α-lipoic acid moiety protruding out of the enzyme's pocket (for a detailed view of the interactions developed between the molecular hybrid and CALB please see supporting information and Fig. S3).

2.3. Enzymatic regioselective synthesis of the gemcitabine-5′-*O*-lipoate (*6*) molecular hybrid and reaction optimization

Having defined *in silico* the capacity of CALB to regioselectively biocatalyze the synthesis of the desired molecular hybrid we then proceeded to the relevant biotransformation. Initially, we screened

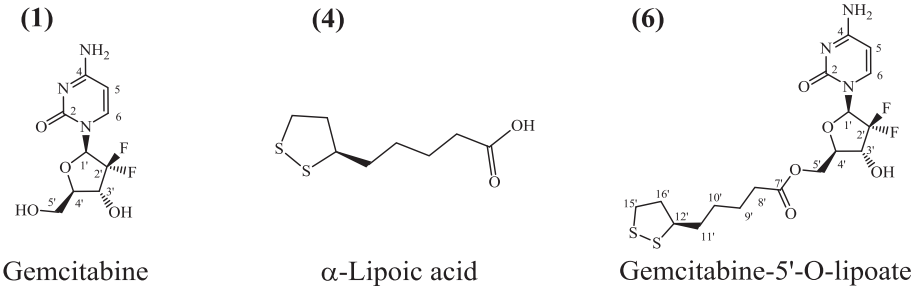

Fig. 1. 2D structures of the antitumor compound GEM (**1**), the antioxidant LA (**4**) and the molecular hybrid gemcitabine-5′-*O*-lipoate (**6**).