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Synthesis of amphiphilic resveratrol lipoconjugates and evaluation of their anticancer activity towards neuroblastoma SH-SY5Y cell line

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ABSTRACT

Resveratrol, a polyphenol present in grapes and other edible plants, possesses several important pharmacological activities, including anticancer activity. Nevertheless, its therapeutic use is still limited because of some unfavourable physicochemical and pharmacokinetic properties, mainly, poor cellular uptake and too rapid metabolism resulting in elimination from the body. To meet these drawbacks, some resveratrol conjugates would be useful, which would possess improved stability, uptake and bioavailability than the lead compound, and the ability to release it once it is internalized into the cell. In this paper we report a synthetic strategy which allowed us to obtain new amphiphilic resveratrol derivatives starting from different selectively protected resveratrol phosphoramidites or even from the resveratrol triphosphoramidite. Specifically, resveratrol was conjugated through phosphate bridge(s) to different lipophilic groups related to membrane lipids, such as cholesteryl or diacylglycero moieties. All the new lipoconjugates were tested towards human neuroblastoma SH-SY5Y cells and proved to be significantly more active than resveratrol, with a concentration-dependent activity.

1. Introduction

E-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, **1**, referred to in this paper as resveratrol) is a polyphenolic phytoalexin synthesized by plants in response to environmental stress or infection [1]. First isolated in 1940 from *Veratrum grandiflorum* [2], it has been later obtained in larger quantities from the roots of *Polygonum cuspidatum* [3], whose preparations, Itadori tea or Ko-jo-kon, have long been used in Japan and China as a traditional remedy for inflammation, allergy and coronary heart diseases (CHD) [4]. Thereafter, resveratrol has been found in grapes, berry, peanuts, and other edible plants as well as in foods and beverages derived from them [5,6], and then it could be considered a common constituent of the human diet.

Since Renaud and de Lorgeril (1992) established a correlation between high red wine consumption and low incidence of CHD [7], there has been a crescendo of literature reports on resveratrol's pharmacological properties that include, besides the above cited cardiovascular protection, antioxidant, anti-inflammatory and antiviral activities, as well as prevention of obesity, diabetes and neurodegenerative diseases [6,8–11]. Furthermore, in the last decade, there has been an explosion of literature about the chemopreventive and chemotherapeutic activities of resveratrol towards cancer; its ability to inhibit the three major stages of

Abbreviations: CHD, coronary heart disease; SULTs, sulfotransferases; UGTs, uridine diphosphate glucuronosyltransferases; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; PMA, phosphomolybdic acid; NB, neuroblastoma; HSF, human skin fibroblasts; QCM-D, quartz crystal microbalance with dissipation monitoring; SLB, supported lipid bilayer; 4′CPR, 4′-*O*-(cholesteryl-3-phosphoryl)resveratrol; 3CPR, 3-*O*-(cholesteryl-3-phosphoryl)resveratrol; 3,5CPR, 3,5-di-*O*-(cholesteryl-3-phosphoryl)resveratrol; 4′DMGPR, 4′-*O*-(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)resveratrol; 3DMGPR, 3-*O*-(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)resveratrol; 4′DPGPR, 4′-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)resveratrol; 3DPGPR, 3-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)resveratrol; 3,5DMGPR, 3,5-di-*O*-(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)resveratrol; 3,5DPGPR, 3,5-di-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)resveratrol; 3,5,4′DMGPR, 3,5,4′-tri-*O*-(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)resveratrol.

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carcinogenesis (initiation, promotion and progression) has been described [12], and its antiproliferative activity towards a wide variety of human tumor cells has been demonstrated in vitro and in vivo in rodents [13]. At the same time, resveratrol safety has been demonstrated in healthy cells [14] and in human clinical trials [15,16].

Unfortunately, resveratrol possesses unfavourable pharmacokinetic properties. Recent studies have shown in rodents and humans that, following oral administration, resveratrol undergoes rapid metabolism by phase II enzymes, specifically SULTs and UGTs, which significantly reduces its bioavailability in the systemic circulation [16]. On the other hand, resveratrol metabolites, even at considerably high concentrations, do not induce tumor cell death or prevent their proliferation in vitro [17,18].

Besides its rapid metabolism, other essential questions about the therapeutic utilization of resveratrol regard its transport in circulating blood and its cell uptake. Because of the poor water solubility of resveratrol, its transport in plasma would be difficult in free form; in fact, to gain access to its targets, resveratrol requires binding to serum proteins such as albumin and lipoproteins [19]. These complexes could be also responsible for the carrier-mediated process in the cellular uptake of resveratrol, for which the implication of both a passive diffusion and a carrier-mediated transport has been demonstrated [20].

In recent years, in order to enhance resveratrol stability, bioavailability and uptake, different biocompatible resveratrol-loaded particles have been developed, using polymeric nanoparticles, liposomes, cyclodextrins, solid lipid nanoparticles or yeast cell as carriers [21]. Most of these drug delivery systems have been tested in vitro [22] and in vivo [23] showing higher activities than free resveratrol. However, despite the positive results obtained in this field, new strategies are still required to achieve an optimal pharmacological response and to enhance resveratrol bioavailability in target cells [16,24].

We report here the synthesis and some biological properties of new amphiphilic resveratrol derivatives where the lead compound has been regiospecifically conjugated through a phosphate bridge to different natural lipophilic moieties selected from those related to membrane lipids.

2. Results and discussion

2.1. Synthesis and characterization of resveratrol lipoconjugates

Amphiphilic lipoconjugates of resveratrol referred to in this study have all the lipophilic moiety anchored, through phosphate bridge(s), at the hydroxyl group(s) of the ring A and/or ring B of the lead compound **1**.

In order to prepare variously structured lipoconjugates, we felt it appropriate to develop first a strategy to synthesize three regioselectively protected resveratrol phosphoramidites as well as the resveratrol triphosphoramidite, to be used later as versatile synthons. Scheme 1 summaries this strategy.

For the synthesis of phosphoramidites **6** and **8**, the key intermediates **3** and **4** were obtained by means of two different enzymatic steps, starting from 3.4′,5-tri-*O*-acetylresveratrol (**2**) or resveratrol (**1**), respectively, and using *Pseudomonas cepacia* or *Candida antarctica* lipases in organic solvents, according to Nicolosi et al. [25]. Phosphoramidite **7** was in turn prepared from the intermediate **5**. The latter was obtained through a regioselective deacetylation of the peracetylresveratrol (**2**) using silica gel as a mild acid catalyst. In practice, a solution of compound **2** in CH_2Cl_2/CH_3OH (2:1) was mixed with SiO_2 and the suspension, evaporated to almost solvent-free condition, was stirred for 62 h at room temperature. Semi-preparative HPLC of the extracted reaction mixture afforded pure 3.4′-di-*O*-acetylresveratrol (**5**, 36% final yield). HPLC profile of the final reaction solution is shown in Fig. 1.

Compound **5** had already been obtained by Torres et al. [26] following enzymatic acetylation of resveratrol catalyzed by immobilized *Alcaligenes sp.* lipase QLG. However, the commercial unavailability of the enzyme has led us to develop the aforementioned method.

The next treatment of protected resveratrol **3**–**5** and resveratrol with the phosphitylating reagent 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite in the presence of DIPEA and successive purification by PLC gave 3,5-di-*O*-acetylresveratrol-4′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (**6**), 4′,5-di-*O*-acetylresveratrol-3-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (**7**), 4′-*O*-acetylresveratrol-3,5-di-[(2-cyanoethyl)-*N*,*N*-diisopropyl] phosphoramidite (**8**) and resveratrol-3,5,4′-tri-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (**9**) in good yield. The structures of phosphoramidites **6**–**9**, which until now have not been reported in the literature, were determined on the basis of their spectroscopic features. ^1H and ^{13}C NMR spectral data are reported in Experimental section. A detailed analysis of ^1H and ^{13}C resonances as well as of ^{13}C–^{31}P coupling constants clearly indicated the presence and the expected position of the phosphoramidite function(s). ESI-MS (+) spectra of both compounds **6** and **7** showed quasimolecular ions at *m*/*z* 513 $[M + H]^+$, 535 $[M + Na]^+$ and 551 $[M + K]^+$, the ESI-MS (+) spectrum of compound **8** showed quasimolecular ions at *m*/*z* 671 $[M + H]^+$, 693 $[M + Na]^+$ and 709 $[M + K]^+$, while the ESI-MS (+) spectrum of compound **9** showed a quasimolecular ion at *m*/*z* 829.4 $[M + H]^+$.

Once phosphoramidites **6**–**9** were obtained, these were reacted with cholesterol or with the appropriate 1,2-di *O*-acylglycerol in order to obtain the desired resveratrol lipoconjugates.

By applying the phosphoramidite chemistry, we firstly accomplished the coupling of phosphoramidite **6** with cholesterol using tetrazole as the activating agent in anhydrous conditions (Scheme 2). Then, after oxidation of the phosphite triester by iodine, all protecting groups were removed by treatment with ammonia, in the presence of sodium metabisulfite as antioxidant agent. Finally, semi-preparative HPLC of the crude reaction mixture afforded pure 4′-*O*-(cholesteryl-3-phosphoryl)resveratrol (4′CPR, **10**) in 52% yield. On the basis of 1D and 2D experiments (see paragraph 4.1.), all the resonances of ^1H and ^{13}C NMR spectra of compound **10** were assigned, as reported in Experimental section. Evidences of the presence and position of the phosphate bridge were provided by very diagnostic signals in the ^{13}C NMR spectrum. Specifically, two doublets at δ 154.1 (J_{COP} = 6.5 Hz) and 121.3 (J_{CCOP} = 5.2 Hz), assignable to C-4′ and C-3′/5′of resveratrol, respectively, and three doublets at δ 77.6 (J_{COP} = 5.8 Hz), 41.5 (J_{CCOP} = 4.4 Hz) and 31.1 (J_{CCOP} = 3.6 Hz), assignable respectively to C-3, C-4 and C-2 of the cholesteryl residue, clearly located the phosphate bridge between C-4′ and C-3 of cholesteryl. Confirmatory evidence of the structure of **10** was provided by its ESI-MS(−) spectrum showing the peak of quasimolecular ion $[M - H]^-$ at *m*/*z* 675.

When the above procedure was performed on the phosphoramidite **7**, 3-*O*-(cholesteryl-3-phosphoryl)resveratrol (3CPR, **11**) was obtained in 57% yield. The structure of compound **11** was determined by means of detailed analysis of its ^1H NMR, ^{13}C NMR and ESI-MS(−) spectra (see Experimental section). The ^1H NMR spectrum revealed the absence of symmetry in ring A, whose 2-H, 4-H and 6-H protons shifted downfield (Δδ = 0.48, 0.41 and 0.20 ppm, respectively) as compared to the corresponding resonances of resveratrol in the same solvent. These data, corroborated by ^{13}C–^{31}P coupling constants in the ^{13}C NMR spectrum located the phosphate bridge between C-3 of resveratrol and C-3 of cholesteryl moiety.

Properly adapting the above reported procedure to the