



A novel hybrid drug between two potent anti-tubulin agents as a potential prolonged anticancer approach

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ABSTRACT

We report the design, synthesis and biological characterisation of a novel hybrid drug by conjugation of two tubulin inhibitors, a hemiasterlin derivative **A** (H-Mpa-Tle-Aha-OH), obtained by condensation of three non-natural amino acids, and *cis*-3,4',5-trimethoxy-3'-aminostilbene (**B**). As we have previously demonstrated synergy between **A** and **B**, we used a monocarbonyl derivative of triethylene glycol as linker (**L**) to synthesise compounds **A-L** and **A-L-B**; via HPLC we analysed the release of its potential hydrolysis products **A**, **A-L**, **B** and **B-L** in physiological fluids: the hybrid **A-L-B** undergo hydrolysis in rat whole blood of the ester bond between **A** and **L** (half-life = 118.2 ± 9.5 min) but not the carbamate bond between **B** and **L**; the hydrolysis product **B-L** was further hydrolyzed, but with a slower rate (half-life = 288 ± 12 min). The compound **A-L** was the faster hydrolyzed conjugate (half-life = 25.4 ± 1.1 min). The inhibitory activity of the compounds against SKOV3 ovarian cancer cell growth was analysed. The IC₅₀ values were 7.48 ± 1.27 nM for **A**, 40.3 ± 6.28 nM for **B**, 738 ± 38.5 nM for **A-L** and 37.9 ± 2.11 nM for **A-L-B**. The anticancer effect of **A-L-B** was evidenced to be obtained via microtubule dynamics suppression. Finally, we stated the expression of the active efflux transporters P-gp (ABCB1) and MRP1 (ABCC1) in the human normal colon epithelial NCM460 cell line by reverse-transcription PCR. Via permeation studies across NCM460 monolayers we demonstrate the poor aptitude of **A** to interact with active efflux transporters (AET): indeed, the ratio between its permeability coefficients for the basolateral (B) → apical (A) and B → A transport was 1.5 ± 0.1 , near to the ratio of talatubulin (1.12 ± 0.06), an hemiasterlin derivative able to elude AETs, and significantly different from the ratio of celiprolol (3.4 ± 0.2), an AET substrate.

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Abbreviations: A, H-Mpa-Tle-Aha-OH (hemiasterlin derivative obtained by condensation of three non-natural amino acids) – Mpa, N-(1-methyl-1-phenylethyl)-(D)-alanine – Tle, (D)-t-Leucine – Aha, (2E, 4S)-2,5-dimethyl-4-(methylamino)-2-hexanoic acid; **B**, *cis*-3,4',5-trimethoxy-3'-aminostilbene; **C**, *cis*-3,4',5-trimethoxy-3'-hydroxystilbene (internal standard for kinetic studies); **L**, monocarbonyl-triethylene glycol; **A-L-B**, hybrid compound obtained by coupling **A** with **B**, using **L** as a spacer; **A-L**, compound **A** linked to the spacer **L** using an ester function; **L-B**, compound **B** linked to the spacer **L** using a carbamate function; AETs, active efflux transporters; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; MDR, multidrug resistance.

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1. Introduction

Some of the most important targets of anticancer drugs are the tubulin-binding sites of microtubules (Jordan and Wilson, 2004; Yue et al., 2010). Vinca alkaloid and taxane derivatives can interact with specific binding sites, while stilbene derivatives are able to interact with the *colchicine* binding site of tubulin (Gupta and Bhattacharyya, 2003; Yue et al., 2010). Interestingly, a new tubulin binding site has been identified for the hemiasterlins, a family of natural tripeptides found in marine sponges (Yue et al., 2010). Among the hemiasterlins, the synthetic derivative *taltubulin* has been characterised as a potent antitumoral agent (Zask et al., 2005).

Taltubulin can be synthesised by condensing three non-natural amino acids, although the enantioselective synthesis of one of these amino acids has been reported to be especially difficult (Nieman et al., 2003; Simoni et al., 2010). Recently, however, a versatile enantioselective means of

synthesising a new class of synthetic hemiasterlins has been proposed (Hsu et al., 2012). The new hemiasterlins [an example, compound **A** (H-Mpa-Tle-Aha-OH) is reported in Fig. 1] have proved to be potent tubulin inhibitors, able to induce a strong synergism with cis-3,4',5-trimethoxy-3'-aminostilbene (compound **B**, Fig. 1) (Simoni et al., 2010).

We report here the synthesis and characterisation of the hybrid compound obtained by coupling hemiasterlin **A** with the stilbene derivative **B**, using the monocarbonyl derivative of triethylene glycol (**L**) as a spacer (compound **A-L-B**, Fig. 1). The hydrolysis potential of this conjugate in physiological fluids (cell culture media, whole blood and liver homogenates) was explored, in order to determine whether it could feasibly act as a controlled release system for the hemiasterlin **A**, whose activity is potentiated by the presence of the stilbene derivative **B**. In order to complete the characterisation, the cytotoxic activity of both the hybrid compound and its hydrolysis products was investigated. Finally, we have evaluated the potential ability of the hemiasterlin derivative **A** (the main active hydrolysis product of the hybrid **A-L-B**) to elude the active efflux transporters (AETs), that on the cell membrane are the body's first line of defence against xenobiotics. These transporters drive substrates across biological membranes, even against concentration gradients, thereby limiting xenobiotic entry into the cells. This important system of defence is generally enormously enhanced in cancer cells, in which overexpression of AETs, most notably the ATP-binding cassette (ABC) efflux transporters ABCB1 (for P-glycoprotein, P-gp), ABCC1 (multidrug resistance-associated protein, MRP1) and ABCG2 (breast cancer resistance protein, BCRP), has been implicated in multidrug resistance (MDR) (Aller et al., 2009). These ABC efflux transporters,

especially ABCB1 and ABCC1, appear to be expressed in a wide variety of cancers (Dean et al., 2001; Wu et al., 2011; Pavan et al., 2014).

In order to characterise the efficacy profiles of novel anticancer drugs, it is therefore vital to consider their interactions with ABC efflux transporters (International Transporter Consortium et al., 2010). Here we report the use of normal human colon epithelial NCM460 cells as a suitable cellular model for efflux studies referred to compound **A**. In particular, by RNA extraction and reverse-transcription PCR, NCM460 cells were shown to express the P-gp and MRP1 active efflux transporters. The permeation studies were completed by comparison of the permeability coefficients of **A** for the basolateral (B) → apical (A) and B → A transport with those of *celiprolol* and *taltobulin*, chosen for their ability to interact with the efflux transporters or elude them, respectively (Karlsson et al., 1993; Loganzo et al., 2003).

2. Materials and methods

2.1. Chemistry

Commercially available solvents and reagents were used without further purification. Reactions and product mixtures were routinely monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates (Merck F254) using the indicated solvent systems. Flash chromatography was carried out with Merck silica gel (230–400 mesh). All the final products undergoing biological testing were purified by preparative reversed-phase HPLC [Waters Delta Prep LC 40 mm assembly column C18 (30 cm × 4 cm, 15 μm particle size)] eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% CH₃CN + 0.1% TFA in

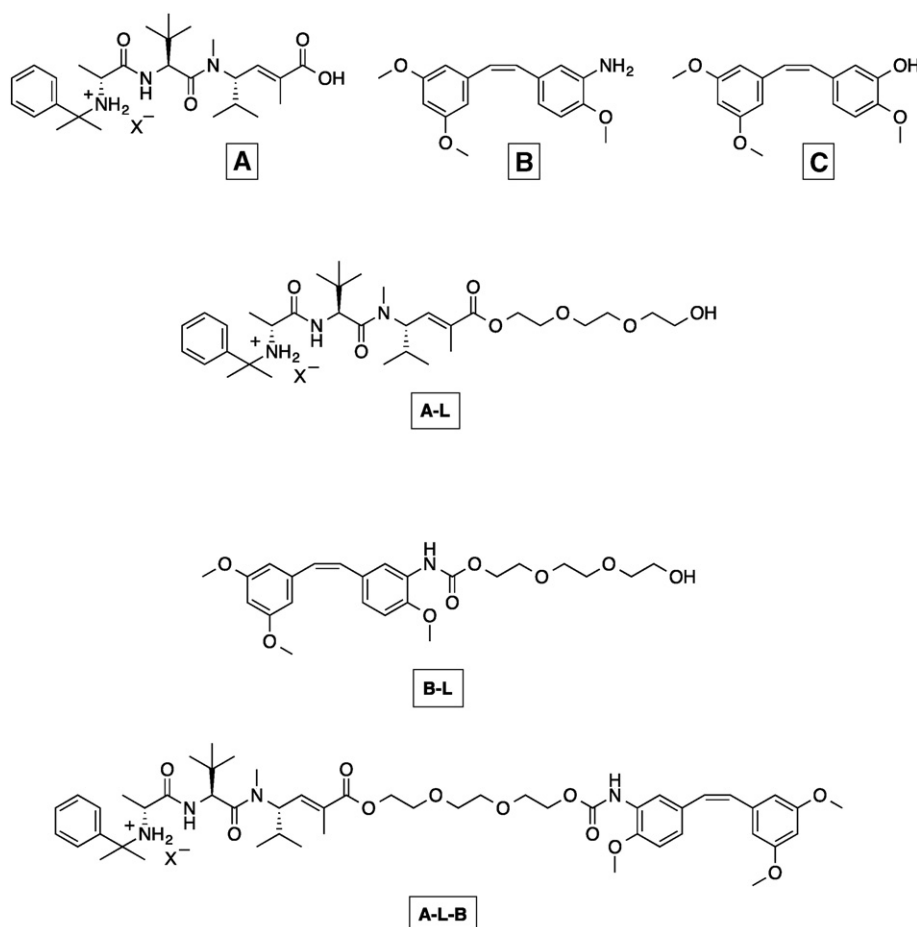


Fig. 1. Chemical formulas of the analysed hemiasterlin (**A**, **A-L**) and stilbene (**B**, **B-L**) derivatives and their hybrid **A-L-B**. The stilbene derivative **C** was employed as an internal standard in HPLC analysis. X⁻ = CF₃CO₂⁻.

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